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ANNUAL REPORT OF THE  
LABORATORY OF BIOCHEMICAL GENETICS  
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE  
October 1, 1987 through September 30, 1988

*Annual Report*

Previously, many neuroblastoma and related somatic hybrid cell lines were shown to acquire voltage-sensitive ion channels and other neuronal properties when intracellular cyclic AMP levels were elevated for a number of days. Cells with elevated cAMP acquire new proteins such as the  $\alpha$ -subunit of voltage-sensitive calcium channels and other proteins of unknown function. A cDNA library was constructed from poly A<sup>+</sup> RNA prepared from NG108-15 neuroblastoma-glioma hybrid cells that had been treated for 5 days with 1 mM dibutyryl cAMP. The library was screened and 17 cDNA clones were obtained that correspond to species of RNA that are 3 to 40 times more abundant in cells treated with dibutyryl cAMP than in cells cultured without this compound. Each cloned cDNA was used as a probe with Northern blots to determine the number of species of poly A<sup>+</sup> RNA responsive to dibutyryl cAMP and the chain length of each species of RNA. The results suggest that the 17 cDNA clones correspond to species of RNA transcribed from 10 genes. Partial nucleotide sequences of the cDNA inserts from 3 clones were obtained. Clone NG-32 corresponds to mouse mitochondrial mRNA for ATP synthase subunit 6, a mitochondrial gene. This protein is part of the H<sup>+</sup> channel portion of the mitochondrial ATP synthase complex. Treatment of NG108-15 cells with dibutyryl cAMP results in an 8-fold increase in the abundance of mRNA for this protein. The nucleotide sequence of clone NG-10 cDNA was identified as part of the D-loop region of mouse mitochondrial DNA that contains the origin of replication for the heavy strand of DNA. The 5'-terminal nucleotide sequence of some molecules of heavy strand mitochondrial DNA is known to consist of a short segment of RNA that is complimentary to a short light strand mitochondrial DNA sequence nearby. Hence, NG-10 cDNA may correspond to an RNA transcript of the light strand of mitochondrial DNA that serves as a primer for the initiation of heavy strand mitochondrial DNA synthesis. Treatment of NG108-15 cells with dibutyryl cAMP results in a 40-fold increase in this species of RNA. These results show that treatment of NG108-15 neuroblastoma-glioma cells with dibutyryl cAMP results in marked increases in the abundance of RNA transcripts from heavy and light strands of mitochondrial DNA. Further work is needed to determine whether cAMP regulates mitochondrial biogenesis or the ability to synthesize ATP.

A  $\lambda$ gt11 cDNA library was prepared from rat brain poly A<sup>+</sup> RNA and screened with oligodeoxynucleotide probes that correspond to the  $\alpha$ -subunit of L-type voltage-sensitive calcium channels. Eleven positive clones were detected that have cDNA inserts 1.6-5.5 Kb in length. Nucleotide sequence analysis reveals strong homology as well as differences in the deduced amino acid sequences of the  $\alpha$ -subunits of rat brain and rabbit skeletal muscle L-type



voltage-sensitive calcium channels.

To detect recombinant DNA clones that correspond to novel homeobox genes a Drosophila genomic DNA library was screened with multiple oligodeoxynucleotide probes, each designed to hybridize to multiple homeobox genes. Five clones that gave positive signals with 2 or more oligodeoxynucleotide probes exhibited specificities that could not be explained on the basis of known nucleotide sequences of Drosophila homeobox genes. Nucleotide sequence analysis of the homeobox regions of 4 clones revealed 4 new homeobox genes (NK-1,2,3,4). Two recombinant clones contained identical DNA inserts, each insert contained 2 new homeobox genes (NK-3 and NK-4). The deduced amino acid sequence of the NK-1 homeobox exhibits the highest homology to the homeobox regions of deformed, zen-2, and zen-1 (75, 72, and 71% homology, respectively). The relative homology of the NK-2 homeobox is as follows: NK-4 > NK-3 > NK-1 = IAB-7. The homology of NK-3 is: NK-2 > labial > NK-4 > NK-1; and NK-4 homology is NK-2 > zen 2 = NK-3 > labial. Genomic DNA fragments from the 4 new homeobox genes were used to screen cDNA libraries prepared from poly A<sup>+</sup> RNA from 0-3 hr Drosophila embryos or from 3 - 12 hr embryos.; One NK-1 cDNA clone was obtained from the 3-12 hr embryo cDNA library, but none was detected in the 0-3 hour embryo library. Comparison of the nucleotide sequences of NK-1 cDNA and genomic DNA clones showed that the NK-1 gene has 3 exons. One of the 2 introns detected resides within the homeobox region.

#### Regulation of rat neuropeptide Y gene expression:

Untreated PC12 rat pheochromocytoma cells and N18TG-2 mouse neuroblastoma cells possess relatively low basal levels of neuropeptide NPY (0.25 and 0.13 pg/ $\mu$ g total RNA), while NG108-15 mouse neuroblastoma x rat glioma hybrid cells contain remarkably high amounts (11 pg/ $\mu$ g RNA). Untreated human neuroblastoma lines SK-BN-SH and SK-N-MC also contain relatively high amounts of NPY mRNA.

During the past year we studied the regulation of NPY mRNA abundance in PC12 cells by cAMP, phorbol esters, glucocorticoids, and calcium ionophore. The results are as follows:

Cyclic AMP elevation by forskolin or 8-bromo-cAMP elicits moderate elevation (4-10-fold) over 12-48 hr of treatment and synergizes with phorbol ester.

Phorbol esters such as phorbol 12-myristate 13-acetate (PMA) that activate protein kinase C elicit little or no effect alone but synergize with cAMP to produce large elevations (20-200-fold) over 12-48 hr of treatment. Responses to phorbol ester are enhanced by A23187, a calcium ionophore, which increases the cytoplasmic Ca<sup>++</sup> ion concentration.

Nerve growth factor (NGF (2.5 S) strongly elevates NPY mRNA (40-100-fold) during 1-6 days of treatment. The increase is evident by as early as 3 hr of NGF exposure and is sensitive to cycloheximide, indicating a requirement for protein synthesis. Phorbol ester synergizes with NGF to produce 300-fold elevations in NPY mRNA.



Glucocorticoids such as dexamethasone (Dex) elicit 2-3-fold potentiations of the cAMP and phorbol-ester elicited elevations of NPY mRNA. Dex biphasically modulates the stimulations by NGF, potentiating early (3-10 hr) effects of NGF but profoundly inhibiting the large stimulations at later times (1-6 days). The latter inhibitory phase resembles previously described antagonisms by glucocorticoids of NGF inductions of specific mRNAs.

We have looked for an effect of NGF treatment on the stability of NPY mRNA. Upon addition of actinomycin D to inhibit RNA synthesis, NPY mRNA in control and NGF-treated cells decayed with half-lives of 5 and 9 hr, respectively. We conclude that part of the increase in NPY mRNA due to NGF is a consequence of increased NPY mRNA stability, but that transcriptional activation must also take place to account for the 40-100-fold elevation of NPY mRNA. Regulation of NPY mRNA levels also was observed in SK-N-MC human neuroblastoma cells, which have a high constitutive expression of the gene. Dex, forskolin, or PMA have no effect alone, but Dex + forskolin and forskolin + PMA double the NPY mRNA level.

#### Regulation of proenkephalin (pEnk) gene expression.

Glucocorticoids and cAMP synergistically increase the abundance of pEnk mRNA in C6 rat glioma cells. We examined the mechanism of this increase by run-on transcription experiments involving nuclei isolated from C6 cells treated with or without Dex and/or forskolin for 1-24 hr. Dex alone had no effect on the pEnk transcription rate, and forskolin alone elicited a brief stimulation that reached 6-fold at 1 hr. Dex + forskolin elicited a more sustained stimulation of 5-6 fold at 2-6 hr. These results suggest that cAMP elevates pEnk mRNA in C6 cells by stimulating transcription and that glucocorticoids exert a permissive effect by sustaining the stimulation by cAMP.

We are presently searching for a putative glucocorticoid regulatory element of the pEnk gene by transient expression assays of plasmids having portions of the pEnk gene linked to a reporter gene, chloramphenicol acetyltransferase (CAT). Rat proenkephalin genomic clones were isolated and mapped. A fragment containing 2500 bases of the 5' upstream region and 47 bases of the first exon was ligated in both orientations in front of the CAT gene in a promoterless vector, and this construct was transfected into C6 rat glioma cells. CAT expression was found to respond as expected to forskolin but did not respond, either with or without forskolin, to Dex. Thus the putative element is not within the sequence tested. Since regulatory elements are sometimes found in introns, another construct that contains the above sequence plus the rest of the first exon and all of the first intron has been constructed and is being tested.

We found that untreated SK-N-MC human neuroblastoma cells contain a surprisingly high abundance of pEnk mRNA. Treatment of the cells with forskolin elevated the level 2-fold. PMA + A23187 markedly reduced the level. Dex did not affect basal or forskolin-stimulated levels but did increase the levels in the presence of the inhibitory combination forskolin + PMA + A23187. Thus, cooperative regulation of the human pEnk gene by cAMP and glucocorticoids exists, as with the rat gene, but is





quantitatively less significant in cells having a high constitutive expression of the gene.

Cytoplasmic components of ACh receptor aggregates - We previously showed that vinculin,  $\alpha$ -actinin and filamin, all of which are cell adhesion associated proteins, are concentrated at the sites of newly formed nictonic acetylcholine (ACh) receptor aggregates. However, the resolution of our techniques was not adequate to determine the localization of proteins within the aggregates. We have devised a novel technique to overcome this problem. Using a selective replating of myoblasts, large myotubes are grown with very few fibroblasts. A coverslip coated with a purified polypeptide adhesive from shellfish is attached to the upper cell surface, where the ACh receptor aggregates have formed. This coverslip is then lifted, with plasma membranes and partially disrupted myotubes attached, allowing access of antibodies to the membrane cytoskeleton, a high degree of spacial resolution in the plane of the membrane, and minimal cytoplasmic background staining.

With this technique, we have found that vinculin and associated actin filament bundles are adjacent to, but not superimposed upon the ACh receptor enriched domains of the aggregates, while a 43 kilodalton protein (the "43K protein", closely associated with ACh receptors in other systems) and actin in another form are precisely colocalized with the receptors. These results suggest the existence of at least 2 distinct membrane cytoskeleton domains within the newly formed ACh receptor aggregates.

These membrane preparations also facilitated the precise localization of clathrin, the major protein of the coated vesicle "basket". Clathrin is not obviously concentrated in ACh receptor aggregates and is excluded from the ACh receptor enriched domains, but most aggregates contain clathrin in the form of tiny speckles, possibly corresponding to coated pits involved in exocytosis or endocytosis.

Immunogold localization of ACh receptors, the 43K protein and sodium channels. We have succeeded in labeling 2 integral membrane proteins (ACh receptors and sodium channels) and the 43K protein at the electron microscopic level by a postembedding immunogold technique. At both the neuromuscular junction (NMJ) and the ACh receptor aggregates formed in culture, the ACh receptors and the 43K protein are precisely colocalized. Sodium channels are concentrated at the NMJ at a lower apparent density than ACh receptors or 43K protein. However, unlike the ACh receptors, which are concentrated at the crests of the postsynaptic membrane folds close to the nerve ending, the sodium channels are distributed throughout the folds. This result confirms, with higher resolution, results previously obtained with the immunoperoxidase technique.

In cultured myotubes, ACh receptors and the 43K protein are also colocalized in apparently intracellular membrane compartments which remain to be characterized.



Escherichia coli the interaction of cAMP with the cAMP receptor protein (CRP) induces a conformational change in the structure of the protein thereby converting it to a form that is active in regulating gene expression. This regulatory protein has been mutated to a form that functions in gene transcription in the absence of added cAMP. It was the purpose of this study to isolate one of these mutant proteins, the NCR91 protein, crystallize it, and determine the X-ray crystallographic structure of the protein for comparison with that of the structure of the wild-type protein.

Since the cAMP synthesizing system in Escherichia coli is regulated by the sugar transport system known as the phosphoenolpyruvate:sugar phosphotransferase system (the PTS), an interest in our laboratory has been to understand the various mechanisms by which the process of sugar transport is regulated. One of the mechanisms that has been described in gram-positive bacteria for regulation of sugar transport is the process of inducer expulsion. When Streptococcus pyogenes are grown under conditions for the induction of the lactose transport system and then allowed to take up a nonmetabolizable substrate for this system (thiomethylgalactoside, TMG), the subsequent addition of glucose elicits the rapid release into the medium of the accumulated TMG. In this organism, the uptake of TMG is via the PTS and it is presumed that the PTS is involved in the expulsion mechanism. It was the purpose of this study to examine the mechanism of sugar transport regulation in an organism that transports sugars by a mechanism other than the PTS. A representative heterofermentative lactobacillus, Lactobacillus brevis was chosen for the study.

The organisms used in the studies were Escherichia coli, Lactobacillus brevis and Lactobacillus buchneri. Cloning the gene for the CRP protein from strain NCR91 of Escherichia coli provided a basis for hyperexpression of the protein. The hyperexpressed protein was purified by a conventional method to produce homogeneous CRP91. Crystals of the protein were grown in the presence of cAMP under the same conditions as for the wild-type protein (in the presence of phosphate buffer at room temperature). Diffraction data on a single crystal were collected on a Nicolet imaging proportional counter and then processed on a VAX computer. A difference Fourier map of the CRP91 and wild-type CRP proteins was calculated using the program PROTEIN. Uptake of thiomethylgalactoside into Lactobacilli was measured using the radioactive sugar analog. Pools of free or phosphorylated thiomethylgalactoside were measured in boiled extracts of cells which were then fractionated by anion exchange chromatography. Cell-free extracts of Lactobacilli were prepared by sonication of cell suspensions. Sugar phosphorylation in cell extracts was measured by incubating the extracts with radioactive thiomethylgalactoside or 2-deoxyglucose.

The Crystal Structure of a cAMP-independent Mutant of the cAMP



Receptor Protein: Escherichia coli NCR91 synthesizes a mutant form of the cAMP receptor protein in which alanine 144 is replaced by threonine. This mutated form of CRP can, in the absence of adenylate cyclase, confer on cells the CRP\* phenotype which is due to the ability of the mutated CRP to function as a transcription regulator in the absence of cAMP. CRP91 has been purified and crystallized with cAMP under the same conditions as used to crystallize the wild-type CRP-cAMP complex. X-ray diffraction data were measured to 2.4-Angstrom resolution and the CRP91 structure was determined using initial model phases from the previously determined wild-type structure. A difference Fourier map calculated between CRP91 and the wild-type CRP showed the two alanine to threonine sequence changes in the dimer and also a change in the orientation of cysteine 178 in one of the subunits. Refinement of the structure indicated that there were small differences in the CRP91 structure compared to that of the wild-type protein that included concerted motions in the small domains, in the hinge region between the two domains and in an adjacent loop between beta-strands 4 and 5. These findings indicate that the mutation at residue 144 causes changes in the position of some protein atoms that are distal to the mutation site.

Regulation of beta-Galactoside Transport and Accumulation in Heterofermentative Lactic Acid Bacteria. Lactobacillus brevis and Lactobacillus buchneri are examples of heterofermentative organisms. They do not have a functional phosphoenolpyruvate:sugar phosphotransferase system and transport thiomethylgalactoside by an active transport mechanism that results in the accumulation of intracellular free thiomethylgalactoside. When cells were preloaded with thiomethylgalactoside and then exposed to glucose, there was a rapid efflux of the intracellular galactoside. When the glucose was depleted from the medium by uptake into the cells, then there was a restoration of the uptake and accumulation of thiomethylgalactoside. The glucose-promoted efflux of thiomethylgalactoside required the intracellular phosphorylation of glucose. The glucose-promoted efflux was not inhibited by iodoacetate. These results were interpreted to indicate that a phosphorylated metabolite of glucose at or above the level of glyceraldehyde-3-phosphate was required to evoke displacement of intracellular thiomethylgalactoside from these cells. Experiments using the counterflow technique indicated that exposure of cells to glucose converted the uptake of thiomethylgalactoside from an active uptake mechanism to a facilitated diffusion mechanism that allowed equilibration of thiomethylgalactoside between the intracellular and extracellular spaces. Since this phenomenon in the heterofermentative Lactobacilli had similar characteristics to that of inducer expulsion that takes place in the homofermentative Streptococcus and Lactobacillus species, a possible similarity in the mechanism was explored. The inducer expulsion mechanism in the homofermentative bacteria involves HPr, a protein component of the phosphoenolpyruvate:sugar phosphotransferase system which appears to serve also as a transport regulator. Using complementation assays with strains of Staphylococcus that are



deficient in HPr, it was established that Lactobacillus brevis extracts have HPr activity although this organism lacks a functional phosphoenolpyruvate:sugar phosphotransferase system. This study is consistent with the idea that HPr can function as a sugar transport regulator independently of its role as a phosphocarrier in sugar transport.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00009-14 LBG

## PERIOD COVERED

October 1, 1987 - September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cell Recognition and Synapse Formation

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Marshall Nirenberg, Chief, LBG, NHLBI  
Hemin Chin, Staff Fellow, LBG, NHLBI  
Li-Shan Hsieh, Visiting Fellow, LBG, NHLBI  
Wu-Hong Tsai, Visiting Fellow, LBG, NHLBI  
Maria Giovanni, Staff Fellow, LBG, NHLBI  
David Trisler, Guest Worker, LBG, NHLBI  
Yongsok Kim, Visiting Fellow, LBG, NHLBI  
Dana Hilt, Staff Fellow, LBG, NHLBI

## COOPERATING UNITS (if any)

Bruce Schrier, LDN, NICHD

## LAB/BRANCH

Laboratory of Biochemical Genetics

## SECTION

Section of Molecular Biology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

10

## PROFESSIONAL:

8

## OTHER:

2

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

1. Treatment of NG108-15 neuroblastoma-glioma hybrid cells results in marked increases in the abundance of certain species of RNA. Seventeen cDNA clones corresponding to these species of RNA were obtained and the nucleotide sequences of three clones were determined. DNA clone NG-32 corresponds to mRNA for ATP synthase subunit 6, which is transcribed from the heavy chain of mitochondrial DNA and codes for a protein that is part of the  $H^+$  channel of the ATP-synthase complex. Clone NG-10 DNA corresponds to another mitochondrial RNA of unknown function, which is transcribed from the light chain of mitochondrial DNA and may be involved in the initiation of replication of mitochondrial heavy strand DNA.
2. Eleven cDNA clones were obtained that correspond to mRNA for the  $\alpha$ -subunit of the L-type voltage-sensitive calcium channel of rat brain. Analysis of the DNA sequence and the deduced amino acid sequence reveals strong homology between brain and skeletal muscle calcium channel  $\alpha$ -subunits. Approximately 75% of rat brain  $\alpha$ -subunit amino acid residues that were defined are either identical to the amino acid residues of rabbit skeletal calcium channel  $\alpha$ -subunit or are conservative amino acid replacements.
3. Four novel Drosophila homeobox genes NK-1, -2, -3, and -4 were cloned and partial nucleotide sequences were determined. One NK-1 cDNA clone was obtained from a cDNA library prepared from poly A<sup>+</sup> RNA from 3-12 hr Drosophila embryos, but none was detected in the 0 to 3 hr embryo library. Six NK-3 cDNA clones were obtained from a library prepared from Drosophila poly A<sup>+</sup> RNA from 0 - 3 hr embryos and 6 additional clones were obtained from a library prepared from 3 - 12 hr embryo poly A<sup>+</sup> RNA. The exon-intron structure of the NK-1 gene was determined. One of the two introns found resides within the homeobox.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00018-11 LBG

## PERIOD COVERED

October 1, 1987 - September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Neuropeptide Gene Expression

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Steven L. Sabol, M.D., Ph.D., Medical Officer (Research), LBG, NHLBI  
Jay Joshi, Ph.D., Senior Staff Fellow, LBG, NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Biochemical Genetics

## SECTION

Section on Molecular Biology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2.0

## PROFESSIONAL:

2.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project includes several studies on the regulation of biosynthesis of protein precursors of neuropeptides in the mammalian nervous system.

One study concerns the regulation of the gene coding for neuropeptide Y (NPY), an important neurotransmitter in the central and peripheral nervous systems. We have studied the effects of hormones and second-messenger systems on the abundance of NPY precursor mRNA in clonal PC12 rat pheochromocytoma cells. Large increases in NPY mRNA levels were elicited by the synergistic actions of cyclic AMP and phorbol esters, which activate protein kinases A and C, respectively. Furthermore, glucocorticoids and the calcium ionophore A23187 potentiate this effect, resulting in NPY mRNA levels up to 200 times the control. Treatment of PC12 cells for 1-6 days with nerve growth factor (NGF) also elevates NPY mRNA 40-100-fold as a result of both transcriptional activation and increased mRNA stability. The action of NGF is profoundly inhibited by glucocorticoids, illustrating an important antagonism between NGF and glucocorticoids in the regulation of neural gene expression.

A second project concerns regulation of transcription of the gene coding for proenkephalin, the precursor of the opioid peptides methionine- and leucine-enkephalin. Glucocorticoids and cAMP synergistically increase the transcription of the proenkephalin gene and the abundance of proenkephalin mRNA in C6 rat glioma cells. Glucocorticoids exert a permissive effect on proenkephalin gene transcription by prolonging the transcriptional stimulation elicited by cAMP elevation. Sequences near and within the rat NPY gene were examined for a glucocorticoid regulatory element by the transient expression of fusion genes, but none was found within 2500 bases upstream from the promoter.

These studies shed light on the control of biosynthesis of peptides that are important in autonomic regulation, pain perception, and cognitive function.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00151-18 LBG

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Biology of Cyclic Nucleotides in E. coli

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A. Peterkofsky Deputy Chief LBG, NHLBI

Others: J. Harman Staff Fellow LBG, NHLBI

P. Reddy Staff Fellow LBG, NHLBI

J. Reizer Staff Fellow LBG, NHLBI

N. Amin Visiting Fellow LBG, NHLBI

COOPERATING UNITS (if any) National Bureau of Standards, Gaithersburg, MD (I. T. Weber);  
The University of Connecticut, Storrs, CT (A. H. Romano)

## LAB/BRANCH

Laboratory of Biochemical Genetics

## SECTION

Macromolecules Section

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

3.4

## PROFESSIONAL:

2.4

## OTHER:

1

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A. Structure-Function Analysis of cAMP-independent Forms of the cAMP Receptor Protein (CRP). The cAMP receptor protein (CRP) ordinarily requires cAMP for function in operon expression. Mutants of CRP, termed CRP\* proteins, have the unique property of functioning in the absence of added cAMP. Sequence analysis of cloned CRP\* DNA has allowed the primary structure of such proteins to be determined and compared with that of the wild-type protein. The gene for one of the cAMP-independent proteins, termed NCR91, has been cloned into a high expression vector; using this construct, it has been possible to grow E. coli under conditions where substantial amounts of CRP91 are formed. The protein has been crystallized and its structure analyzed by X-ray diffraction. This data has permitted a comparison of the crystal structure of the mutant protein with that of the wild-type protein.

B. Regulation of Sugar Transport in Gram-positive Bacteria. Regulation of sugar metabolism in gram-positive bacteria is uniquely different from that in gram-negative bacteria, because gram-positive bacteria don't make cAMP. In gram-negative bacteria, the adenylate cyclase system is regulated by the phosphoenolpyruvate:sugar phosphotransferase system (PTS). Some gram-positive bacteria transport sugars by the PTS; this sugar transport system also plays a role in the process of inducer expulsion whereby the addition of glucose to cells loaded with a nonmetabolizable sugar triggers the elimination of that sugar. Some strains of the gram-positive lactobacilli do not have an active PTS but nevertheless show the process of inducer expulsion. The characteristics of the inducer expulsion process in Lactobacillus brevis was studied and it was demonstrated that, although there is not a functional PTS in this organism, one of the components of the sugar transport system is present. It is suggested that this component may play a role in regulating sugar transport in Lactobacillus brevis.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00153-01 LBG

## PERIOD COVERED

October 1, 1987 - September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Differentiation of the Postsynaptic Cell Membrane

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Mathew P. Daniels Research Biologist LBG-NHLBI

Others: Bernhard E. Flucher Special Volunteer LBG-NHLBI

Fanjie Zeng Special Volunteer LBG-NHLBI

## COOPERATING UNITS (if any)

R. Bloch, University of Maryland Medical School, Dept. of Physiology

## LAB/BRANCH

Laboratory of Biochemical Genetics

## SECTION

Section on Molecular Biology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

3

## PROFESSIONAL:

2 1/4

## OTHER:

3/4

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Our goal is to study the cellular and molecular mechanisms involved in the assembly, remodeling and elimination of chemical synapses. Our primary approach is to study the formation, stabilization and elimination of nicotinic acetylcholine receptor aggregates on striated embryonic muscle fibers grown in tissue culture. The formation of these receptor aggregates is induced by neuronal factors present in embryonic brain extract. The acetylcholine receptor aggregates, together with associated cell surface structures, provide a model of postsynaptic differentiation at the skeletal neuromuscular junction.

We use fluorescence and electron microscopy, as well as immunocytochemistry to follow changes in the distribution of acetylcholine receptors and associated cell surface components, and to study the underlying ultrastructural changes.

In the past year, we have made the following observations:

1. Newly formed acetylcholine receptor aggregates contain at least two distinct types of membrane-cytoskeletal domains:  
a) receptor enriched domains, associated with a 43 kilodalton protein and actin. b) "adhesion" domains, associated with vinculin, and actin filament bundles.
2. Newly formed acetylcholine receptor aggregates also contain punctate sites of clathrin immunoreactivity, possibly representing coated pits.
3. As seen by immunogold labeling, the 43 kilodalton protein is precisely colocalized with acetylcholine receptors at the crests of the postsynaptic folds in the neuromuscular junction, and in acetylcholine receptor aggregates formed in culture. In contrast, sodium channels are distributed throughout the postsynaptic membrane folds of the neuromuscular junction, at an apparently lower site density than the acetylcholine receptors at the crests.





Annual Report of the  
Laboratory of Biochemistry  
Section on Enzymes  
National Heart, Lung, and Blood Institute  
October 1, 1987 to September 30, 1988

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Role of Oxygen-Mediated Inactivation of Enzymes in Protein Turnover, Aging,  
Neutrophil Function, and Oxygen Toxicity

Chronic and acute oxygen toxicity is implicated in a growing list of pathologic processes which include arthritis, the aging process, carcinogenesis, broncho-pulmonary dysplasia and adult respiratory distress syndrome, retinopathy of prematurity, and reperfusion-mediated ischemic damage. Oxidative modification of proteins has been demonstrated in some of these disorders and likely occurs in all of them. Oxidative modification might also function physiologically; for example, in controlling the switch from anaerobic to aerobic metabolism, in host defense mechanisms, in limitation of the inflammatory response, and in some mechanisms of protein turnover.

(1) Role of Protein Oxidation in Aging. Using the amount of protein carbonyl groups as a measure of protein damage due to MFO-catalyzed reactions, it was established that the level of oxidized protein which accumulates in rat hepatocytes increases with the age of the animals over the range of 2 to 26 months. This increase in oxidized protein is accompanied by the accumulation of altered forms of glutamine synthetase and glucose-6-P dehydrogenase as disclosed by: (a) a progressive loss in specific catalytic activity, (b) a progressive increase in the amount of immunologically cross-reactive protein per catalytic unit, (c) a progressive increase in the fraction of heat labile enzyme activity, (d) a progressive increase in the carbonyl content of glutamine synthetase protein.

A role of MFO-catalyzed oxidation reaction in the age-related accumulation of oxidized enzymes is supported by the fact that similar changes occur when young rats are exposed to 100% oxygen for up to 48 hours (see last year's annual report). The age-related, and oxygen stress-provoked accumulation of oxidized proteins is correlated with a progressive decrease in the level of cytosolic neutral protease activities, one of which was shown earlier to preferentially degrade the oxidized forms of enzymes. The results underscore the importance of maintaining balance between the rate of protein oxidation on the one hand and the rate of degradation of oxidized protein on the other. In the case of oxygen stress, the oxidized protein which accumulates during the first 48 hours of exposure to oxygen is subsequently degraded (between 48 and 56 hours) in response to an increase in neutral protease activity. In contrast, during aging, the level of neutral protease continues to decline with age, suggesting that the ability to increase the level of neutral protease to compensate for the increase in level of oxidized protein has been compromised.

(2) Diet Restriction and Germ-Free Animal Studies. Rat testis from conventionally maintained animals and from germ-free animals were obtained from the Lobund aging studies. These tissue preparations were used to study the effects of diet restriction and animal age on the levels of oxidized protein (protein carbonyl groups) and glutamine synthetase activity.



In contrast, the level of oxidized protein in testis from germ-free animals increased only slightly with age and was not affected by diet restriction. In all cases, the specific catalytic activity of glutamine synthetase varied inversely with the level of oxidized protein, suggesting that the loss of activity is associated with oxidation modification.

(3) Oxidative Inactivation of Glutamine Synthetase. E. coli glutamine synthetase serves as an excellent model for detailed investigations of the mechanism of protein modification by MFO systems. Results of earlier studies with the ascorbate/Fe(III)/O<sub>2</sub> MFO system indicated that inactivation of the enzyme is associated with the modification of one histidine residue in each subunit of the enzyme and that the modification of two histidine residues renders it susceptible to proteolytic degradation. A more detailed investigation of the kinetics of these processes shows that the enzyme is inactivated when an average of only 0.5 of a histidine residue has been modified per subunit. This implies either that subunit interactions are involved in the inactivation reaction or that another more rapid oxidative modification is involved.

When glutamine synthetase is oxidized by the ascorbate-MFO system, about 0.5 of ascorbate per subunit is tightly bound to the enzyme at a site near to the divalent metal binding site in the catalytic site of the enzyme. It was demonstrated, however, that inactivation of the enzyme is not due to ascorbate binding; ascorbate binds to the enzyme only after it has been inactivated.

Absorption difference and second-derivative rapid scanning spectroscopies, polyacrylamide gel electrophoresis and the sensitivity to proteolysis by trypsin have been used to probe the conformational and structural changes which occur during the oxidation of glutamine synthetase by MFO systems. It was shown that oxidation of the enzyme leads to an increase in polarity of the environment surrounding tyrosine residues and that this increase in polarity is accompanied by changes in the kinetics and thermodynamics (i.e., the melting temperature, enthalpy, activation energy) of a spectroscopically detectable protein thermal transition, and also to an increase in sensitivity of the enzyme to proteolysis by trypsin. With prolonged exposure of glutamine synthetase to MFO systems, it undergoes significant fragmentation. The fragmentation patterns obtained with several different MFO systems are qualitatively the same, suggesting that certain peptide bands are particularly sensitive to oxidative cleavage.

(4) Identification and Quantitation of Oxidized Amino Acid Residues in Protein. To facilitate the identification of the amino acid residues of proteins which are oxidized by MFO systems, the carbonyl groups which are formed during MFO treatment were converted to the corresponding chromophoric secondary amine derivatives of either p-aminobenzoic acid or fluoresceinamine, via reduction of their Schiff base intermediates with cyanoborohydrides. From spectrophotometric analysis of the chromophoric adducts of the p-aminobenzoic acid derivatives, it was demonstrated that the oxidation of homopolymers leads to significant peptide bond cleavage; poly-L-histidine was particularly susceptible to oxidative fragmentation. The fluoresceinamine derivative of  $\gamma$ -glutamylsemialdehyde was identified as a major product of oxidized glutamine synthetase and is produced also from the oxidation products of homopolymers of arginine, proline, and lysine.

(5) Amino Acid Peroxidation. A number of iron-containing compounds of biological origin were found to replace synthetic iron chelates in catalyzing the



peroxidation of amino acids. Detailed studies with a system comprised of hydrogen peroxide, bicarbonate, hemin or ferritin was found to mimic the bicarbonate-dependent oxidation of leucine by the more conventional Fenton reagent (hydrogen peroxide plus Fe(III) plus EDTA). A complete analysis of the reaction products established that leucine oxidation occurs by three independent pathways: (1) Oxidative deamination to form alpha-ketoisocaproic acid and nitrogen; (2) Oxidative decarboxylation-deamination to form isovaleraldehyde, bicarbonate and nitrogen; (3) Oxidation to the isovaleraldehyde oxime and carbon dioxide. Material and oxidation-reduction balances demonstrate that these three reactions and the amino acid independent iron-catalyzed decomposition of hydrogen peroxide to oxygen account for 90-95% of the hydrogen peroxide and leucine which are consumed. By means of difference spectroscopy, it was shown that there is a time-dependent interaction between Fe(III), oxygen and amino acid. This together with results of earlier studies showing that amino acid oxidation is insensitive to radical scavengers supports the conclusion that amino acid oxidation involves in situ generation of an activated oxygen species within the Fe(III)- or Fe(II)-amino acid complex, and is not the result of a subsequent free-radical attack of the amino acid via oxygen radicals generated by an amino acid independent mechanism.

(6) Role of MFO Systems in Regulation of Metabolic Pathways. Studies of Klebsiella aerogenes demonstrate that oxidative modification of enzymes can function as a specific regulator of metabolic pathways, even though the oxidation is presumably mediated by a free radical reaction. When switched from an anaerobic to an aerobic environment, there is an immediate repression of synthesis of several key enzymes including glycerol dehydrogenase, ethanol dehydrogenase, and 1,3-propanediol oxidoreductase. Other dehydrogenases are constitutive, including malate, glucose-6-P, and isocitrate dehydrogenases. However, the cell does not simply rely on repression to control the level of enzymes. The three enzymes whose syntheses are repressed are also rapidly inactivated. This oxidative inactivation requires protein synthesis. However, exposure of the cells to micromolar concentrations of hydrogen peroxide also inactivates the susceptible enzymes, without protein synthesis. The constitutive dehydrogenases are unaffected by the peroxide, and cell viability is unchanged. Thus, the switch from anaerobic to aerobic metabolism may be mediated by a site-specific free radical oxidation which utilizes hydrogen peroxide. Protein synthesis may be required to generate an oxidase for the production of hydrogen peroxide, which then functions as a kind of "second messenger."

(7) AIDS Research. The fact that MFO systems catalyze oxidative modification of proteins and nucleic acids at metal binding sites is the basis of an effort to develop site-specific oxidizing reagents for the inactivation key enzymes in the human immunodeficiency virus. Results of preliminary experiments show that the reverse transcriptase of this virus can be oxidatively inactivated by MFO systems comprised of either ascorbate, copper and O<sub>2</sub> or dithiothreitol, iron and O<sub>2</sub>. The viral protease has been isolated in an inactive form from a fusion product produced in E. coli by means of recombinant technology.

The autooxidation or peroxidation of EDTA·Fe(II) chelate complexes leads to the generation of hydroxyl radicals or other active oxygen species that can cause scission of DNA strands. This principle has been used in an attempt to synthesize highly specific inhibitors of HIV. To achieve specificity for the cleavage of HIV DNA, EDTA was conjugated to a 28 mer phosphorothiolate oligodeoxynucleotide



(S-ODN) which was complementary to a sequence in the tatIII region (6001-6031) of HIV DNA. It is presumed that the synthetic EDTA-oligonucleotide conjugate will react preferentially with the HIV DNA. Consequently, hydroxyl radicals generated at the EDTA·Fe(II) site will be targeted for cleavage of the HIV DNA within the tat region. To facilitate binding to DNA, the intercalator, acrylidine, was attached to the 5'-end of the oligonucleotide.

Results of preliminary studies carried out in collaboration with Drs. Stein and Mari of the Clinical Pharmacology Branch of NCI have shown that the EDTA-S-ODN and S-ODN were equally effective in inhibiting the cytopathic effects of HIV infected T4 cells; moreover, the S-ODN-EDTA analog was less effective than S-ODN in the inhibition of gag protein synthesis in chronically HIV-infected cells. Further studies in which the effects of Fe(II) and/or H<sub>2</sub>O<sub>2</sub> are examined will be needed to establish the efficacy of the targeted EDTA.

(8) Regulation of Nitrogen Metabolism in E. coli. In earlier studies, it was observed that the ability of high concentrations of ammonia to repress the synthesis of glutamine synthetase in E. coli is overridden by the presence of several D-amino acids. It was determined that the amino nitrogen of these D-amino acids is used for the synthesis of L-serine, probably via an increase in the level of serine hydroxymethyltransferase (SHMT) activity. In continuing studies, it was established that strains of E. coli carrying mutations in glyA, the structural gene for SHMT, are deficient in their ability to elicit the D-amino acids response. The D-amino acid response was eliminated also when the D-amino acids are added together with L-serine, adenine (but not hypoxanthine), glutamine, azaserine, or trimethoprim. This raises the possibility that enhancement SHMT activity by D-amino acid might lead to a diversion of formyl groups toward serine synthesis at the expense of other metabolic pathways (e.g., the synthesis of purine). Whatever the mechanism, the D-amino acid response is under control of the glnB gene product (P<sub>II</sub>-protein) which is implicated in the regulation of glutamine synthetase activity as well as its synthesis. In the absence of the glnB gene product, the D-amino acid response, as well as the capacity to increase glutamine synthetase under nitrogen-limited growth conditions, is abolished.





Annual Report of the  
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The research projects of the investigators in the Section on Metabolic Regulation are mainly concerned with the physical, chemical, and biological approaches to resolve the mechanisms of enzyme action and its regulation. In the past year, research has been concentrated on (1) investigating the mechanisms of signal and energy transduction, particularly those involving phosphoinositides and those inducible by oscillating membrane potentials; (2) studying the role of multienzyme complexes in the transfer of metabolites in the glycolytic pathway; (3) elucidating the mechanism of enzyme action and regulation which includes Ca(II)-calmodulin-dependent protein phosphatase, phosphoinositide-specific phospholipase C, and Ca(II)-sensitive protein phosphatase; and (4) developing analytical methods for general biomedical research. Together these projects will provide a better understanding of how enzymes in living cell function and are regulated.

I. Signal and Energy Transduction.

A. Signal Transduction Involving Phosphoinositides.

1. Characterization of Phosphoinositide-specific Phospholipase C and Its Isozymes. Phosphoinositide-specific phospholipase C (PLC) plays a major role in biological signal transduction because it catalyzes the hydrolysis of phosphoinositol biphosphate ( $PIP_2$ ) to generate two second messenger molecules, inositol trisphosphate ( $IP_3$ ) and diacylglycerol (DAG), in response to a transmembrane signal.  $IP_3$  induces the release of Ca(II) from endoplasmic reticulum, while DAG activates protein kinase C. Dr. Rhee and his coworkers have purified three immunologically distinct phosphoinositide-specific phospholipase C (termed PLC-I, II, and III) from bovine brain. To sequence these isozymes, PLC-related cDNA clones corresponding to PLC-I, II, and III were isolated from a rat brain gt 11 expression cDNA library using specific monoclonal antibodies. Complete cDNA sequences for PLC-I, II, and III yielded 5209, 5106, and 2769 base pairs, which deduced to a protein of 1215 ( $M_r=138,225$ ), 1289 ( $M_r=148,431$ ), and 756 amino acids ( $M_r=85,840$ ), respectively. The determination of open reading frames was abided by the amino acid sequences of 22, 21, and 9 tryptic peptides isolated from bovine PLC-I, II, and III, respectively. Amino acid and nucleotide sequences for bovine brain PLC-II and III were also determined. Comparative analysis of the amino acid sequences of the isozyme from bovine brain and rat brain showed 96.3% identity for the complete sequence of PLC-II, 89% for the partial sequence (lacks 61 amino acids at N-terminal) of PLC-III, and 95% identity for the 28 amino acid sequence for PLC-I. However, nucleotide sequence conservation is lower, with 87.9% identity for PLC-II and 84.8% for PLC-III, due to the presence of a large number of silent mutations. The relatively low conservation found in PLC-III amino acid sequence is consistent with the immunological data obtained with monoclonal antibodies derived against bovine isozymes. All 6 anti-PLC-I antibodies and 22 out of 23 anti-PLC-II antibodies cross-reacted with their



respective rat isozymes while 4 out of 12 anti-PLC-III antibodies failed to recognize rat PLC-III.

Comparative study of the three PLC isozymes reveals that there exists an overall sequence homology in two major regions which are separated by a variable region and that the length of this intervening region is different for PLC-I, II, and III. The two homologous regions, termed regions A and B, are comprised of about 150 and 120 amino acids, respectively. These two regions are separated by 71, 487, and 50 amino acid residues in PLC-I, II, and III, respectively. It is believed that regions A and B probably constitute, either separately or jointly, the basic domain(s) responsible for the catalytic function, such as specific binding of phosphoinositides, the hydrolysis of phosphodiester bonds, or for the interaction with a yet unidentified G-protein. It is highly significant that two regions of PLC-II, domain C (amino acid residues 555-598) and domain D (residues 668-705) show a high degree of amino acid similarity to the products of various tyrosine kinase-related oncogenes (yes, src, fgr, abl, fps, fes, and tck). Both domains C and D are located in the variable region which separates the two conserved domains A and B. In tyrosine kinases, the homologous domains C and D are located in the region which is not essential for the protein tyrosine kinase activity, but they are likely to be involved in an interaction with cellular components which modulate the function of these kinases. This amino acid similarity suggests that PLC-II and cytoplasmic tyrosine kinases may be regulated by a common cellular component(s). On the other hand, PLC-I and III do not contain the C and D domains and their variable regions are very different. Thus, the three isozymes are likely regulated differently and respond to different external signals.

2. Purification and Characterization of IP<sub>3</sub>-3-Kinase. Inositol-1,4,5-trisphosphate (IP<sub>3</sub>) is a second messenger for Ca(II) mobilization from intracellular stores in response to external signals. The fate of IP<sub>3</sub> is determined by two enzymes, an IP<sub>3</sub>-3-kinase, which converts inositol-1,4,5-trisphosphate to inositol-1,3,4,5-tetrakisphosphate, and a 5-phosphomonoesterase, which converts IP<sub>3</sub> to inositol-1,4-bisphosphate. The IP<sub>3</sub>-3-kinase activity in crude extracts of bovine brain can be resolved into two peaks, and each of these two fractions further purified on Matrix green affinity gel and calmodulin affinity column. Although the enzyme preparations were not completely homogenous, the molecular weights as determined by SDS-PAGE appeared to be 95,000 for one activity and 53,000 for the other activity. The 95K enzyme was activated about six-fold by calmodulin with an  $S_{0.5}$  ~10 nM in the presence of 0.2  $\mu$ M Ca(II), whereas the 53K enzyme appeared to be insensitive to calmodulin. Both enzymes showed a  $K_m$  of 0.7  $\mu$ M for IP<sub>3</sub>. The high affinity of these isozymes for IP<sub>3</sub> together with their differential response to Ca(II)/calmodulin suggests that these isozymes may play an important regulatory role in inositol phosphate signaling by generating additional inositol polyphosphate isomers which can modulate additional cellular events.



## B. Effect of Oscillating Membrane Potentials on the Dynamics of Biological Signal and Energy Transduction.

1. Theoretical Treatment. Fluctuations and periodic oscillations of physical and chemical parameters are inherent to the environment of many proteins, particularly those embedded within the membranes of living cells. When the fluctuations induce changes in transmembrane potential, a modest amplitude of these fluctuations will lead to changes in electric field strength large enough to produce a shift in conformational equilibria of many membrane proteins. A theory has been formulated for understanding the effect of oscillations and energy-driven fluctuations on the environmental parameters of the catalytic activities of enzymes, particularly those embedded within the biological membrane. Enzyme catalysis is coupled to the environment by interaction between the catalytic transitions and thermodynamic parameters such as temperature, pressure, and local electric field strength. Much previous work has stressed only the influence of the environment on the free energy of the enzyme-catalyzed reaction. In the present work, the effects of environmental parameters on the conformational transitions intrinsic to the protein are also emphasized. It is the possibility of such an interaction which is one important factor distinguishing enzymes from small molecule catalysis. The treatment given applies in principle to any catalyst even in homogeneous solution. However, the nonlinear terms giving rise to free-energy transduction would typically be negligibly small. It is the conformational flexibility of proteins, and the positioning of certain enzymes within the bilayer where local electric field strength fluctuations may be as high as  $10^7$  V/cm, which provides the significant expression of this fundamental nonlinearity. When conformational transitions of the protein are tied to catalytic events at the active site, this could lead to significant enhancement of enzyme efficiency by coupling to the energy-driven fluctuations inherent in the environment. The theoretical analysis reveals that the nonlinearity can allow an enzyme of sufficient complexity to transduce free energy from the source of the fluctuations to do work by driving either a chemical reaction or transport of substance across a membrane against its chemical gradient specified by the  $\Delta G$  of the process. Free-energy transduction can occur even if the output reaction is itself not directly influenced by the fluctuating parameter. In addition, free energy transduction from a dynamic perturbation with a time average of zero can be accomplished via the fact that flexible macromolecules can absorb energy from oscillating external fields into conformational degree of freedom, which in turn varies the catalytic activity of an enzyme to allow the conservation of some of this energy by doing work on an output reaction. The development of this theory provides an explicit extension of irreversible thermodynamics to the case of nonstationary environments. The results highlight the role of proteins as free energy converters as well as biological catalysts, and provide understanding on how a biological organism makes use of energy-driven fluctuations for the purpose of signal and free energy transduction and how enzymes are capable of transducing this inflowing energy into a form usable in metabolism.
2. Experimental Approach. We have designed and constructed a device for applying oscillating electric fields with a variable amplitude up to  $\pm 150$  V/cm



and variable wave form to cell suspensions. The apparatus can monitor the transient conductance of both sample and reference cell and the data are stored in a computer for subsequent analysis. Comparison of the Fourier spectrum of the input periodic potential and the output current signal will allow for detection and analysis of nonlinear responses of membrane processes to stimulation. With this device one can directly determine rate coefficients for a variety of membrane processes induced by electric field.

## II. Mechanism of Enzyme Action and Regulation

### A. The Role of Multienzyme Complexes in Metabolite Transfer in the Glycolytic Pathway.

Srivastava and Bernhard proposed that glycolytic enzymes form multienzyme complexes for direct transfer of metabolites from the producing enzyme to the utilizing one (*Science* **234**, 1081; *Current Topics in Cellular Regulation* **28**, 1). The proposed model differs from the normally assumed mechanism where metabolite is transferred from one enzyme to the next enzyme by means of dissociation and diffused randomly through the aqueous media. Since the direct transfer mechanism does not involve the aqueous environment, the energetics of metabolite conversion can differ from that predicted based on data obtained in aqueous solution. Among the experimental evidence reported in support of the proposed model are: (i) Transient kinetic evidence suggests that NADH is transferred directly from one dehydrogenase to another since the rate coefficients for the transfer of NADH are relatively constant ( $\sim 142\text{--}232\text{ sec}^{-1}$ ), particularly in the case of  $\alpha$ -glycerol-3-phosphate dehydrogenase (GPDH) where  $k_{\text{off}}$  was reported to be  $9\text{ sec}^{-1}$ , yet  $k_{\text{transfer}}$  for NADH from GPDH to lactate dehydrogenase (LDH) is about  $140\text{ sec}^{-1}$ . In other words, the rate coefficient for direct transfer of NADH from GPDH to LDH exceeds the rate coefficient for GPDH•NADH dissociating into the aqueous media. (ii) The fluorescence titration of GPDH•NADH with excess LDH leads to an estimated distribution of 65% of the NADH ligated to LDH at LDH saturation. (iii) The  $k_{\text{cat}}$  for LDH-catalyzed hydrogenation of pyruvate by GPDH•NADH was determined to be  $\sim 50\text{ sec}^{-1}$ , while  $k_{\text{off}}$  for NADH from GPDH is  $9\text{ sec}^{-1}$ . (iv) The  $K_{\text{m}}$  for dihydroxyacetone phosphate (DHAP) in GPDH-catalyzed reaction is  $200\text{ }\mu\text{M}$ . However, with added micromolar concentration range of aldolase, the  $K_{\text{m}}$  was reduced to  $8\text{ }\mu\text{M}$ . The reduction in  $K_{\text{m}}$  was attributed to the formation of aldolase-DHAP complex which will then form a complex with GPDH to allow the direct transfer of aldolase-bound DHAP to the active site of GPDH.

In view of the fundamental importance of the proposed model, we reinvestigated the evidence for direct transfer of NADH between its complexes with GPDH and LDH, and that for the transfer of DHAP from aldolase to GPDH. Our results indicate that Srivastava and Bernhard's conclusions were largely based on the misinterpretation of the experimental data, although some minor corrections of their data are also necessary. The data revealed that: (i) The rate of displacement of NADH by NAD from its binding site on GPDH is strongly dependent on pH. At pH 7.4 the rate constant for the dissociation is 60 and  $42.4\text{ sec}^{-1}$  determined at  $25^\circ$  and  $10^\circ\text{C}$ , respectively. A rate constant of  $9\text{ sec}^{-1}$  as reported by Srivastava and Bernhard was obtained if the pH of the NAD solution was not adjusted from 3.46 to 7.4. With a value of  $60\text{ sec}^{-1}$  for  $k_{\text{off}}$  it is unnecessary for Srivastava and





Bernhard to invoke direct transfer of NADH from GPDH to LDH in order to explain a  $k_{\text{cat}}$  of  $\sim 50 \text{ sec}^{-1}$  determined for LDH-catalyzed hydrogenation of pyruvate by GPDH•NADH. (ii) Stopped-flow experiments on LDH-catalyzed reactions with GPDH and NADH and 10 mM pyruvate as reactant yielded a  $k_{\text{obs}}$  value of  $\sim 20 \text{ sec}^{-1}$ , while a value of  $\sim 190 \text{ sec}^{-1}$  was obtained in the absence of GPDH. (iii) The high  $k_{\text{obs}}$  relative to  $k_{\text{off}}$  for NADH from GPDH•NADH, obtained for NADH transfer from GPDH to LDH is due to the fact that LDH has a lower affinity for NADH relative to GPDH. At relatively low LDH concentration, only a fraction of GPDH would be displaced at equilibrium, therefore the half-time for approaching this equilibrium would be smaller than that for complete displacement. Hence,  $k_{\text{obs}}$  for the displacement reaction will be greater than the intrinsic  $k_{\text{off}}$ . Proper treatment of the kinetics and equilibrium data for the transfer of NADH between GPDH and LDH indicates that NADH transfer proceeds via a free-diffusion mechanism instead of a direct transfer by means of a ternary complex. (iv) Steady-state kinetic experiments show that LDH is an inhibitor for the GPDH-catalyzed reaction by reducing the concentration of free NADH. Similarly, aldolase inhibits the GPDH-catalyzed reduction of DHAP to glycerol-3-phosphate by binding to the substrate, DHAP. The  $K_{\text{diss}}$  for this complex was determined to be  $14 \mu\text{M}$ , which is in reasonable agreement with the reported affinity of aldolase-DHAP complex which has a  $K_{\text{diss}}$  range from 3 to  $30 \mu\text{M}$ . (v) With the  $K_{\text{diss}}$  determined for GPDH•NADH, one can computer-curve fit the fluorescence titration of GPDH•NADH with LDH reported by Srivastava and Bernhard. The data fit well with a  $K_{\text{diss}}$  of  $1.4 \mu\text{M}$  for halibut muscle LDH•NADH complex, a value within the range of  $1\text{-}3 \mu\text{M}$  reported for skeletal muscle LDH•NADH. Although our experiments do not tell us anything about the possible complex formation between GPDH and LDH in the presence or absence of their substrates, they show that whether such complexes are formed or not, the two enzymes act quite independently. The transfer of NADH occurs via nucleotide in free solution rather than as a unimolecular transfer in a ternary complex.

#### B. Mechanism of Ni(II) Activation of Calcineurin in the Absence of Calmodulin.

The Ca(II)/calmodulin (CaM)-dependent protein phosphatase (CPP) also known as calcineurin is a Zn(II) and Fe(III)-containing enzyme. It requires an additional divalent metal ion for structural stability and full catalytic activity. Of the divalent metal ions studied, Ni(II) is the most potent activator, especially when p-nitro-phenyl phosphate (PNPP) serves as the substrate. In the presence of CaM, two Ni(II) can bind to CPP, in an apparent ordered manner. Binding of the first Ni(II) gives rise to strong activation, while binding of the second Ni(II) results in severe deactivation. In the absence of CaM, CPP is also activated by Ni(II) provided Ca(II) is present. Kinetic analysis reveals that the mechanism of this activation is different because the two Ni(II) can bind in somewhat random fashion. In the proposed mechanism, binding of the first Ni(II) converts CPP to a highly activated form and it also protects the enzyme from thermal denaturation; while binding of the second Ni(II) converts the enzyme to a form of moderate activity and it also prevents the slow conversion of the highly active Ni(II)•CPP to a low-activity form. The proposed mechanism is consistent with the following observation: (i) Despite the fact that binding of Ni(II) to CPP is essentially irreversible, when Ni(II) is below 1 mM, the steady-state rate of dephosphorylation decreases with lower Ni(II) levels. (ii) The time course of Ni(II) activation exhibits an initial lag



phase and this lag time of activation ( $1/k_{\text{obs}}$ ) is nonlinear when plotted against  $1/[\text{Ni(II)}]$ . (iii) Contrary to the  $[\text{Ni(II)}]_2 \bullet \text{CPP} \bullet \text{CaM}$  complex, the rate of  $\text{Ni(II)}_2 \bullet \text{CPP}$  deactivation decreases as  $\text{Ni(II)}$  concentration increases.

#### C. Intrinsic Dissociation Constants for the Four Ca(II) Binding Sites on Calmodulin.

The stoichiometric dissociation constants for  $\text{Ca(II)}$  binding to calmodulin (CaM) has been previously determined at  $25^\circ\text{C}$ , pH 7.0, in the presence of 5 mM  $\text{Mg(II)}$ . The four stoichiometric dissociation constants so determined are 7.5, 2.7, 31, and 31  $\mu\text{M}$  for  $K_1$ ,  $K_2$ ,  $K_3$ , and  $K_4$ , respectively. These constants indicate an initial positive cooperativity followed by negative cooperativity among the binding sites. While these constants adequately describe the interactions between  $\text{Ca(II)}$  and CaM regardless of the binding mechanism, each constant is a composite number whose mathematical expression does vary with the mechanism. Recent x-ray crystallography, NMR, and binding studies on CaM and its fragments revealed that the CaM molecule comprises two regions connected by a long helical rod, each region containing two  $\text{Ca(II)}$  binding sites. In the absence of the target protein, the two sets of sites, designated as the low-affinity (N-terminal) and high-affinity (C-terminal) sites, do not affect each other's binding as evidenced by the observation that the overall CaM- $\text{Ca(II)}$  binding curve is the same as the integrated binding curve for the N- and C-terminal fragments. Based on the available information, the binding of  $\text{Ca(II)}$  to CaM can be represented by a scheme that consists of nine CaM species and four intrinsic dissociation constants with  $K_1'$  and  $K_2'$  assigned for the two high-affinity  $\text{Ca(II)}$  sites, and  $K_3'$  and  $K_4'$ , for the two low-affinity  $\text{Ca(II)}$  sites. Based on this scheme, the mathematical expressions which also account for the statistical factors, can be derived to relate the stoichiometric dissociation constants and the intrinsic dissociation constants. The values of  $K_1'$ ,  $K_2'$ ,  $K_3'$ , and  $K_4'$  were calculated to be 20, 1.1, 62, and 14  $\mu\text{M}$ , respectively. Several interesting facts can be deduced from these constants: (i) The high-affinity sites are highly cooperative, with a Hill coefficient of 1.62, and the low-affinity sites are less cooperative, having a Hill coefficient of 1.35. (ii) The partial negative cooperativity observed for  $\text{Ca(II)}$  binding to CaM is apparently arising from the existence of two independent sets of sites. (iii) The initial binding of  $\text{Ca(II)}$  to both sets of sites is comparable ( $K_1' = 20 \mu\text{M}$  vs.  $K_3' = 62 \mu\text{M}$ ). It is the extent of cooperativity that separates the high and low affinity sites. (iv) Since  $\text{Ca(II)}$  binding to the CaM-phosphodiesterase complex shows only positive cooperativity, the two sets of sites cannot be noninteracting in the presence of the target enzyme. In fact, the low-affinity sites must acquire a much higher affinity for  $\text{Ca(II)}$ , i.e., undergo more dramatic conformational changes, as dictated by the huge increase in the complex's affinity for  $\text{Ca(II)}$ .

#### D. Purification of Ubiquitin-Activating Enzyme and Isopeptidase.

Ubiquitin, a 76-residue ( $M_r=8565$ ) heat-stable protein is known to conjugate proteins via the  $\epsilon$ -amino groups. It has been proposed that protein ubiquitination may play a role in DNA transcription and in ATP-dependent protein turnover in eukaryotes. Ubiquitination of proteins is catalyzed by three proteins, E1, E2, and E3. Protein E1 catalyzes activation of ubiquitin, E2 is a ubiquitin carrier protein and some of its isozymes can catalyze the conjugation reaction, and E3 is the ligase enzyme. Currently, we are purifying E1 from rabbit reticulocytes and from



human erythrocytes. Purification procedures include DEAE-cellulose and ubiquitin Sepharose chromatography for the rabbit enzyme, while in the case of human E1, gel filtration and Cibacron Blue affinity chromatography were used. The purity of human E1 is less than 50% and the purity of E1 from rabbit is still under determination. Purification of isopeptidase has been slow due to the unsuccessful chemical synthesis of ubiquitin-lysozyme conjugate which is needed to monitor this enzymic activity.

#### E. Purification and Characterization of Ca(II)-Sensitive Phosphatases from Bovine Brain.

Several phosphatases from bovine brain, whose activities are affected by Ca(II), have been identified by using various substrates to assay fractions collected at different stages of protein separation. Using p-nitrophenyl phosphate (PNPP) as substrate, a Ca(II)-inhibited phosphatase has been purified to near homogeneity. This enzyme is a small molecular-weight phosphate hydrolase. It does not hydrolyze phosphorylated histones. Since several inositol phosphatases have been shown to hydrolyze PNPP and are inhibited by Ca(II), the possibility that the Ca(II)-inhibited phosphatase may be an inositol phosphatase was tested by (i) using [<sup>3</sup>H]inositol-1,4,5-triphosphate as substrate and (ii) using D-myo-inositol-1,4,5-triphosphate as inhibitor against PNPP as substrate. The experiments failed to detect any significant catalysis or inhibition, suggesting that it is not a 5' or 1' phosphatase. The ability of this phosphatase to catalyze the hydrolyses of other inositol phosphates, however, has not been examined. The molecular weight of the 80% pure enzyme gave values of 52,000-54,000 with SDS gel electrophoresis and 52,000 with Superose 12 in FPLC. It appears that our Ca(II)-inhibited phosphatase is not identical with a similar enzyme reported by Li et al. (*FASEB J.* 2, 1470) which has a substantially smaller molecular weight of 34,000.

By using histone 2B (phosphorylated by cAMP-dependent kinase) as the substrate, we detected a different Ca(II)-inhibited phosphatase which elutes differently from the PNPP phosphatase on Affigel blue and DEAE-cellulose columns. Whether this phosphatase is the same as the one reported previously which dephosphorylates synapsin I and calcineurin cannot be examined at this stage of purity.

#### III. Immunological Study of Ubiquitin and Ubiquitin-Conjugated Proteins.

Antibodies to ubiquitin and its BSA conjugates have been raised in sheep. These antibodies were purified by immunoaffinity adsorption chromatography. This treatment yielded milligram quantities of anti-ubiquitin IgG. Western blotting experiments using crude extracts of various tissues from rat, cow, and human showed that the affinity-purified antibodies are highly specific and do not cross-react with other proteins. The titer of the affinity-purified antiserum ranges from 1:10,000 to 1:10,000,000, depending on the batch. Western blotting with the affinity-purified antiserum raised against ubiquitin-protein conjugates failed to detect ubiquitinated proteins in crude extracts from eleven different rat organs and bovine eye lens. However, under the experimental conditions, the lower limit of detection is 35 micrograms. This sensitivity was improved by enriching the endogenous ubiquitin-protein conjugates using immunoaffinity adsorption chromatography. Crude lysate was passed through a column packed with anti-ubiquitin IgG linked to agarose beads.



Ubiquitin-protein conjugates in the lysate bind to the antibodies and can be eluted in a concentrated solution. Thus, it permitted detection of endogenous ubiquitin-protein conjugates from rat blood lysate which were not detectable by direct Western blotting. This method provides a means for differentiating the two types of ubiquitin-protein conjugates--namely, those linked by isopeptide bonds and those linked by thioester bonds. Currently, we are carrying out immunocytochemical analysis of thin sections of rat brain as a preliminary for studying brain tissue of Alzheimer's patients.

#### IV. Kinetics and Mechanism of Fe(II)(Ferrozine)<sub>3</sub> Formation from Fe(II)(amino acid)<sub>3</sub>.

Covalent modification of amino acid side chains by mixed-function oxidation reactions has been shown to be involved in marking proteins for degradation, aging, and neutrophil action by Stadtman and co-workers. Among the mixed-function oxidation systems, Fenton reagent [Fe(II) or III and H<sub>2</sub>O<sub>2</sub>] has been shown to catalyze nearly quantitative conversion of amino acids (AA) to NH<sub>3</sub> and a mixture of aliphatic aldehydes and carboxylic acids. The oxidation of amino acids is stimulated by the addition of limited chelators such as HCO<sub>3</sub><sup>-</sup>, EDTA, ferrozine (Fz), etc. This suggests that Fe(AA)<sub>n</sub>(chelator)<sub>m</sub> is a preferred complex for amino acid oxidation. When the chelator is Fz, one can detect spectrophotometrically the "mixed" complex formation which is followed by complete displacement of the amino acids by ferrozine, and only the "mixed" complex and not the Fe(II)(Fz)<sub>3</sub> is readily oxidized by H<sub>2</sub>O<sub>2</sub>. Using rapid kinetic methods we have carried out detailed kinetic studies for the formation of Fe(II)(Fz)<sub>3</sub> from Fe(II)(Gln)<sub>3</sub>. Based on the results a stepwise mechanism was proposed and rate constants assigned. In addition, the spectrum of a reaction intermediate was identified.

#### V. Analytical Method.

##### A Modified Continuous Variation Method (Job Plot) for the Determination of Stoichiometry and Affinity Constants for Cooperative Binding Systems.

The Fe(II)-Fz system was used as a model to study the binding mechanism and conditions leading to anomalous binding stoichiometry in a highly cooperative system. The complex(es) formation was monitored spectrophotometrically and two different series of experiments were carried out. In the first series, Fe(II) and Fz concentrations were expressed in their molarities and the total reactant concentration was varied from 10.5 to 210 μM. The apexes of the Job plots consistently yielded a stoichiometry of 3. This indicates that either the three Fz bind to Fe(II) in a one-step manner or the total concentration used is much greater than all of the dissociation constants involved. In the second series, Fe(II) concentration was expressed in terms of its number of binding sites and the total reactant concentration was varied from 7 to 105 μM. The data gave a series of curves whose apexes shifted from a stoichiometry of one to nearly three as the total reactant concentration was decreased. This observation confirmed that the mechanism of binding was the infinite cooperative type as predicted by the theoretical analysis. The shifting stoichiometries indicate that anomalous values may be obtained at low total reactant concentrations when reactant concentrations are expressed in terms of binding sites. Computer curve fitting of the data also indicates that the mechanism is most consistent with a strong positive cooperativity with a K<sub>d</sub> of 7.9 μM. When the data were treated by Hill's plot, it yielded a K<sub>d</sub> of 8.2 μM and a Hill coefficient of 2.9. These experiments demonstrate that in Job plot anomalous





situations exist in binding studies involving cooperativity. Therefore, it is necessary to perform the Job plot experiments with a wide range of total ligand and enzyme concentration in order to obtain the correct stoichiometry and to gain knowledge of the mode of binding.



Annual Report  
Section on Intermediary Metabolism and Bioenergetics  
Laboratory of Biochemistry  
National Heart, Lung, and Blood Institute  
October 1, 1987 to September 30, 1988

In both eukaryotes and prokaryotes there are a few enzymes that are now known to contain essential selenocysteine residues that are incorporated as highly specific components in the polypeptide structures. The cotranslational insertion of these residues is directed by the opal stop codon UGA. In a number of laboratories efforts are underway to elucidate the mechanism of this unusual usage of the UGA codon and to define the factors that are required for selenocysteine incorporation rather than the usual termination function. One aspect of our work in the general area of selenium biochemistry has involved a collaborative effort with August Böck's group in München and with Dolph Hatfield in the National Cancer Institute, NIH, to show that a special tRNA is required to deliver the selenocysteine residue for incorporation at the UGA-specified site. Our experience in identifying trace amounts of selenocysteine in proteins is used to show that <sup>75</sup>Se-labeled selenocysteine is indeed present as the esterified amino acid on the new tRNA.

A considerable effort was made to develop an *in vitro* protein synthesis system from *Escherichia coli* that would identify some of the factors required in the special use of the UGA codon. However, neither a direct translation assay nor a combined transcription-translation system yielded significant product for further analysis of the factors required. Now that the new *E. coli* tRNA gene has been inserted in a multicopy plasmid and the tRNA is available in increased amounts, it can be added as a supplement to the *in vitro* transcription-translation system to see if this was one of the limiting factors.

A project is underway to isolate the gene encoding clostridial selenoprotein A from *Clostridium sticklandii* in order to develop a protein synthesizing system that may be easier to dissect in terms of mechanism of usage of the UGA codon for selenocysteine insertion into proteins. The glycine reductase selenoprotein A chemical properties are much superior in many respects to those of formate dehydrogenase of *E. coli* for detailed gene expression studies. Isolation of the gene also will allow sequence determination of the remainder of the protein molecule and aid in elucidation of the catalytic role of this protein in the reductive deamination of glycine and the associated synthesis of ATP. A revised procedure was developed in which a Clostridial gene library of 1500 to 3000 base pair DNA fragments obtained by digestion with Hind III was inserted into a pUC-13 expression vector and cloned into a compatible *E. coli* strain. Using oligonucleotide probes complimentary to the amino acid sequence of a peptide derived from selenoprotein A, a 2300 base pair fragment that hybridized to the probe was detected in the Hind III digest. Analysis of the cloned pUC-13 expression vector constructs detected 15 clones that contained the same 2300 base pair insert. Digestion of this selected fragment to generate smaller pieces of DNA gave a 400 base pair fragment recognized by the probe. This has been sub-cloned into bacteriophage M 13 in order to generate single-stranded DNA suitable for DNA sequencing. Polyclonal antibodies to selenoprotein A are available for screening of lysed colonies provided the protein is expressed.

*E. coli* formate dehydrogenase, in common with several other enzymes produced by facultative bacteria, requires anaerobic conditions for its expression. In a few instances



it has been reported that bacterial genes expressed under anaerobic conditions require the activity of the enzyme DNA gyrase which increases the supercoiling of DNA. To study factors controlling the anaerobic expression of formate dehydrogenase in E. coli, fusions of the gene for the selenium-containing polypeptide of this enzyme with the  $\beta$ -galactosidase structural gene were used as an easily detectable system. In contrast to certain other enzymes, it was found that inhibition of DNA gyrase activity greatly increased the expression of formate dehydrogenase as judged by measuring  $\beta$ -galactosidase activity. Three different chemical inhibitors of DNA gyrase, one of which is the antibiotic coumermycin, and also a temperature-sensitive mutant of the gyrase, all gave rise to elevated enzyme levels in these experiments. The nature of the factor(s) that reflect the redox level of the cell and detect the extent of supercoiling of the DNA, presumably by binding and influencing transcription, are still unknown.

Replacement of the essential selenocysteine residue in a formate dehydrogenase of E. coli with a cysteine residue has been achieved by site-directed mutagenesis in Böck's laboratory in München. For this the UGA codon that specifies selenocysteine was changed to UGU and to UGC that encode cysteine. Procedures were developed here for purification of both forms of the enzyme in order to compare the relative effectiveness of a selenol versus a thiol as a redox center in an enzyme. The sulfur enzyme is estimated to be 10 or at most 20% as active as the selenium form. Both enzymes are extremely oxygen labile and difficult to isolate. All procedures were carried out in the NIH anaerobic laboratory.

Analysis of the pathway of selenium incorporation into the selenoamino acids, selenocysteine and selenomethionine, has been made using mutants defective in synthesis both of specific selenium-dependent enzymes and seleno-tRNAs. A Salmonella typhimurium mutant (Sel A) and an Escherichia coli mutant (Sel D) were analyzed. Since both of these organisms were shown to contain selenocysteine and selenomethionine in numerous proteins (presumably inserted nonspecifically), the overall pathway up to the point of introduction of selenium into selenoamino acids appears normal. To explain a single genetic lesion as affecting selenation both of tRNAs and selenoenzymes, a missing specific selenium donor may be a possibility.

Selenium-modified tRNAs occur in the populations of several anaerobic bacteria, E. coli and S. typhimurium. So far only 5-methylaminomethyl-2-selenouridine has been unequivocally identified. This selenated pyrimidine is formed in intact tRNAs by removal of sulfur from the corresponding thiol pyrimidine and substitution of selenium. To facilitate the identification of other selenium-modified pyrimidines which may be analogs of known thiol pyrimidines, the synthesis of 5-carboxymethylaminomethyl-2-selenouracil was undertaken. Although an improved procedure was developed that gave a good yield of this selenouracil, attempts to introduce the ribityl group to give the corresponding selenonucleoside were unsuccessful. Since the nucleoside derivatives are the products that are obtained by enzymic digestion of tRNAs, authentic samples are needed for comparisons with isolated samples from biological sources for purposes of identification.

A continuing project on the enzymic mechanism of the anaerobic conversion of acetate to methane and carbon dioxide is part of our research on the biological generation of the hydrocarbon methane and the role of vitamin B<sub>12</sub> compounds in methane fermentations. Soluble enzyme preparations of Methanosarcina barkeri produce methane from the methyl group of acetate and also can accumulate S-methyl-thioethane sulfonate (methyl-S-CoM) in the presence of ATP and hydrogen. Both methane formation from acetate and methylation of S-CoM are stimulated by coenzyme A which indicates that acetyl-CoA is the activated form of acetate required for cleavage of the carbon-



carbon bond. A working hypothesis for the conversion of acetate to methane and carbon dioxide requires the participation of carbon monoxide dehydrogenase as acceptor of the carbonyl group and a corrinoid protein as acceptor of the methyl group. Carbon monoxide dehydrogenase has been purified to homogeneity from M. barkeri and the pure enzyme is used as a supplement for various types of treated extracts. A methyltransferase that transfers the methyl group from methyl-B<sub>12</sub> to thioethane sulfonate to form methyl-SCoM has also been isolated in pure form. This enzyme described earlier by van der Meijden et al. in The Netherlands was purified and shown to participate in the conversion of methanol to methane. Our finding that comparable levels of this enzyme are present in acetate-grown cells of M. barkeri and its virtual absence in Methanosarcina vannieli which uses neither acetate nor methanol indicates a common methyl group transfer step in the pathways of methanol and acetate conversion to methane. The enzyme purified from acetate-grown cells is a 37,000 dalton monomeric protein. It shows a high affinity for HSCoM ( $K_m = 86 \mu M$ ) but the  $K_m$  for free methylcobalamin is high (13.5 mM) suggesting the actual substrate may be a protein-bound methylcobalamin. Supplementation of unfractionated extracts with purified carbon monoxide dehydrogenase or with purified methyltransferase failed to stimulate the overall rate of methane or methyl-SCoM generation from acetate showing that under these conditions neither enzyme was rate limiting. Use of antibodies elicited to these purified enzymes as inhibitors and purification of the putative corrinoid protein acceptor of the acetate methyl group should aid in further analysis of the enzymic mechanism of acetate cleavage.





Annual Report of the  
Laboratory of Biochemistry  
Section on Protein Chemistry  
National Heart, Lung, and Blood Institute  
October 1, 1987 to September 30, 1988

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The Section on Protein Chemistry is studying the physical and chemical properties of macromolecules of biological interest and the roles of ligand binding and of protein-protein and inter- and intra-subunit interactions in enzyme catalysis and regulation. The energetics of ligand binding to proteins involve contributions from both ligand-protein and protein-protein interactions. Ligand-promoted changes in protein-protein interactions underlie the phenomenon of cooperativity in ligand binding to proteins and, in addition, give rise to many examples of stabilization and destabilization of protein structures by ligands and metal ions.

Glutamine synthetase, a strictly regulated enzyme in Escherichia coli, is a dodecamer; each subunit (51,814  $M_r$ ) contains a catalytic site with two essential divalent cation sites ( $n_1$  and  $n_2$ ) and a tyrosyl residue that is the site of covalent modification by enzymatically-catalyzed adenylation-deadenylation reactions. The 12 identical subunits of the enzyme are arranged in 2 superimposed hexagonal rings  $\sim 140$  Å in diameter and centers of adjacent subunits are  $\sim 45$  Å apart. Interactions of divalent cations, substrates, substrate analogues, and inhibitors with glutamine synthetase from E. coli have been studied.

The very tight binding of 2  $Mn^{2+}$ , L-methionine-S-sulfoximine-phosphate, and ADP ( $K'_A > 10^{12} M^{-1}$ ) to each subunit of E. coli glutamine synthetase at pH 7 after phosphorylation of the L-glutamate analogue by ATP stabilizes inter-subunit bonding domains. Various analogues of ATP that are substituted at the 6- or 8-position of the adenine ring are substrates for the phosphorylation of L-met-S-sulfoximine and thereby can be introduced specifically into active sites of the enzyme as structural probes. The distance between active-site nucleotide probes of the enzyme has been measured by fluorescence energy transfer, taking advantage of the essentially irreversible binding of various ADP analogues at neutral pH when bound with L-met-S-sulfoximine-phosphate and  $Mn^{2+}$  at active sites. Previously, we reported that active-site nucleotide probes on the dodecamer are widely separated ( $> 56$  Å) and that energy transfer occurred from a single donor to 2 or 3 acceptors on adjacent subunits. The results obtained in this laboratory are entirely consistent with the atomic model for glutamine synthetase proposed by D. Eisenberg's group at UCLA from X-ray crystallographic analysis (Almasy et al, Nature 323, 304-309, 1986). An unusual feature of the enzyme structure is that the 12 active sites are formed at heterologous interfaces between subunits within a hexagonal ring of the two face-to-face eclipsed rings. The two  $Mn^{2+}$  at an active site are in the C-terminal domain, whereas the nucleotide binding site is in the N-terminal region (near Lys 47) of an adjacent subunit. This explains how the binding of substrates stabilizes inter-subunit contacts of the enzyme. The distance between  $Mn^{2+}$  ions at neighboring active sites within the same ring of six subunits is  $\sim 45$  Å (Almasy et al.) which is less than that estimated between nucleotide probes by fluorescence energy transfer measurements. Apparently, the adenosine moiety of an ATP molecule is more towards the exterior surface of the enzyme than are



bound  $Mn^{2+}$  ions. In attempts to solve this and other question prompted by the proposed atomic model, we have introduced an electron dense nucleotide·Pt(II) complex at either active sites or adenylylation sites and these enzyme derivatives have been sent to D. Eisenberg for crystallization and X-ray analysis. Interestingly, several highly regulated enzymes have been shown to have active sites formed across inter-subunit contacts of oligomeric proteins. Possibly, this provides the necessary evolutionary pressure to select for regulation as well as for catalytic activity of key metabolic enzymes which require oligomeric structures for modulation of activity expression.

Temperature-induced UV spectral changes of the  $Mn^{2+}$ - or  $Mg^{2+}$ -form of E. coli glutamine synthetase in 100 mM KCl are reversible at pH 7.3 and involve the exposure of 1 of the 2 Trp residues/subunit and 2 of the 17 Tyr residues/subunit. Monitoring either Trp or Tyr exposure, the data conform to a 2-state model of unfolding with  $\Delta H_{VH}$  values of ~100 kcal/mol (where the cooperative unit is most likely the dodecamer) but midpoints of the transitions ( $T_m$ ) differed significantly. These observations suggest that different domains of the oligomeric structure unfold independently. Increasing  $[Mn^{2+}]$  from 3.6 to 49  $\mu M$  increased  $T_m$  from 319 (322) to 326 (329) K for Trp (Tyr) exposure; further increasing  $[Mn^{2+}]$  from 0.05 to 10 mM, however, caused a decrease in both  $T_m$  values to 315 (316) K, indicating preferential interactions between  $Mn^{2+}$  and the partially unfolded enzyme at  $>0.1$  mM  $Mn^{2+}$ . Added substrate (Gln, ADP, Gln + ADP, or L-Met-sulfoximine) to Mn·GS increased the  $T_m$  value to a varying extent by preferential binding to the folded form. In fact, the transition state complex  $GS \cdot (Mn_2 \cdot ADP \cdot L\text{-Met-S-sulfoximine-phosphate})_{12}$  was stable in the folded form at least to 345 K. Moreover, an Arrhenius plot of  $V_{max}$  in  $\gamma$ -glutamyl transfer was linear from 276-345 K with  $E_A = 17.4$  kcal/mol. Trp or Tyr exposure of the inactive apoenzyme did not change on heating. Thus, the thermally induced transitions of dodecameric Mn·GS appear to involve the melting of active site structures.

The stacking of glutamine synthetase dodecamers ( $M_r$  622,000) is being studied by calorimetry in order to investigate forces governing macromolecular assembly reactions.  $Zn^{2+}$  binds to a site distinct from the active site of each subunit with  $K'_A = 5 \times 10^6 M^{-1}$  at pH 7.0 and deforms the enzyme in such a way that when 50 mM  $MgCl_2$  also is present, spontaneous face-to-face aggregation of enzyme dodecamers occurs. A dominant role of water in the stacking process is suggested by the large negative value of  $\Delta C_p$  obtained. Thermodynamic parameters for active-site ligand binding to glutamine synthetase also have been determined by calorimetry and equilibrium binding measurements.

Our studies of active-site ligand and metal ion interactions with mammalian glutamine synthetase from bovine brain were summarized in last year's annual report.

The metallochromic indicator 4-(2-pyridylazo)resorcinol (PAR) has been used at pH 7.0 to monitor the mercurial-promoted  $Zn^{2+}$  release from E. coli aspartate transcarbamoylase (ATCase;  $c_{6r6}$ ) and from regulatory dimers ( $r_2$ ) after separating these subunits from catalytic trimers ( $c_3$ ). As previously reported, the properties of PAR- $Zn^{2+}$  interactions make PAR a generally useful



reagent for studying  $\text{Zn}^{2+}$  release from proteins. For example, stopped-flow measurements in the presence of 10-20-fold excess mercurial and 50-fold excess PAR at 500 nm of mercurial-promoted release of  $\text{Zn}^{2+}$  from regulatory subunits (with  $\text{Zn}^{2+}$  tetrahedrally bound by the 4 thiol groups of each r chain) was first-order with a half-time of  $\sim 8$  ms at pH 7.0,  $20^\circ\text{C}$ . This rate is much faster than the corresponding rate of  $\text{Zn}^{2+}$  release from intact ATCase in which  $\text{Zn}^{2+}$  is bound near regulatory:cataytic chain (r:c) contacts. This and other observations indicate that  $\text{Zn}^{2+}$  bonding domains in isolated  $r_2$  subunit are much more accessible to mercurial attack than are  $\text{Zn}^{2+}$  clusters in the intact ATCase molecule. The rebinding of  $\text{Zn}^{2+}$  to aporegulatory subunits was studied using  $(\text{PAR})_2\text{Zn}$  as the donor, but complex equilibria and possibly a rate-limiting release of  $\text{Zn}^{2+}$  from PAR are involved. Nevertheless, a lower limit estimate of  $k = 8 \pm 3 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$  at pH 7.0,  $15^\circ\text{C}$  for the second-order rate constant for  $\text{Zn}^{2+}$  binding to  $r_2$  was obtained. This value indicates that  $\text{Zn}^{2+}$  binding to  $r_2$  is not rate-limiting in ATCase assembly *in vivo*. More recently, we have explored the use of fluorescent  $\text{Ca}^{2+}$  indicators (indo-1 and quin-2), which are EGTA analogues, to obtain the affinity constant for  $r_2$  subunit binding  $\text{Zn}^{2+}$ . Both indo-1 and quin-2 have high affinities for  $\text{Zn}^{2+}$  in 1:1 complexes and are good spectrophotometric probes for  $\text{Zn}^{2+}$  and both indicators competitively remove  $\text{Zn}^{2+}$  from  $r_2$  subunit. After determining the association constants of these indicators for  $\text{Zn}^{2+}$  and spectral changes produced by  $\text{Zn}^{2+}$  binding, an equilibrium constant for  $\text{Zn}^{2+}$  binding to regulatory chains in  $r_2$  subunits was determined:  $K'_A \cong 2 \times 10^{12} \text{ M}^{-1}$  at pH 7.0,  $20^\circ\text{C}$  corresponding to  $\Delta G = -16.5 \text{ kcal/mol}$ . These indicators do not remove  $\text{Zn}^{2+}$  from intact ATCase which is consistent with the fact that EDTA is known to remove  $\text{Zn}^{2+}$  from  $r_2$  subunits but not from ATCase. The indicator indo-1 was used also to assess the effects of allosteric effectors on  $r_2$  binding  $\text{Zn}^{2+}$ : 1 mM CTP and MgATP (1 mM) increased the  $K'_A$  value  $\sim 1.2$  and  $1.7$ -fold, respectively, whereas ATP without 1 mM  $\text{MgCl}_2$  had almost no effect on the affinity of  $r_2$  for  $\text{Zn}^{2+}$ . Various mutants with single amino acid replacements in r chains (received from H. K. Schachman's laboratory) will be examined for changes in the affinity for  $\text{Zn}^{2+}$ , using the same techniques as used for studying  $\text{Zn}^{2+}$  interactions with wild-type  $r_2$  subunits.

The methods developed during our studies on  $\text{Zn}^{2+}$  interactions with ATCase and isolated  $r_2$  subunits will be applied also to our investigations of other biologically important  $\text{Zn}^{2+}$  binding proteins. Mammalian alcohol dehydrogenase and transcriptional factor TFIIIA from frog Xenopus laevis (responsible along with at least two other factors for directing the synthesis of oocyte 5S RNA genes) are two  $\text{Zn}^{2+}$  binding proteins that can be studied by the techniques developed in this laboratory. Since TFIIIA is a member of a growing list of important DNA-binding proteins with characteristic structures involving a large number of " $\text{Zn}^{2+}$  fingers" (9-11), it is important to study the structure-function relationships of this small protein ( $M_r$  38,000).



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00202-17 LB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Kinetics, Regulation, and Mechanism of Biochemical Reactions

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	P. Boon Chock	Chief, Section on Metabolic Regulation	LB, NHLBI
Others:	R. Dean Astumian	Staff Fellow	LB, NHLBI
	James Cook	Staff Fellow	LB, NHLBI
	Rixin Zhou	Guest Worker	LB, NHLBI
	Luz Hermida	Guest Worker	LB, NHLBI
	Yi-Xiang Chen	Volunteer	LB, NHLBI
	Charles Y. Huang	Research Chemist	LB, NHLBI

## COOPERATING UNITS (if any)

T.Y. Tsong, Johns Hopkins University School of Medicine, Baltimore, MD; D. Yang, Georgetown University, Washington, DC; H. Gutfreund, Fogarty Scholar-in-Residence, (Bristol University, Bristol, England).

## LAB/BRANCH

Laboratory of Biochemistry

## SECTION

Section on Metabolic Regulation

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

4.85

## PROFESSIONAL:

4.25

## OTHER:

0.60

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

(1) A direct transfer mechanism for metabolites from the producing enzyme to the utilizing one via multienzyme complexes has been proposed for the glycolytic pathway. In view of its fundamental importance, we reinvestigated the evidence for direct transfer of NADH between its complexes with  $\alpha$ -glycerol-3-phosphate dehydrogenase (GPDH) and with lactate dehydrogenase (LDH). Our results revealed that the proposed direct transfer of NADH between GPDH and LDH is mainly based on a misinterpretation of the experimental data, although some minor corrections of their data are also necessary. (2) A theory for explaining the effect of oscillations and energy-driven fluctuations on the environmental parameters on the catalytic activities of enzymes, particularly those embedded within cell membranes, has been formulated. The ability of proteins to absorb energy from oscillating external parameters into their conformational degree of freedom was emphasized. The theory provides a reasonable mechanism for energy transduction, even when the transduction is derived from a dynamic perturbation with a time average of zero. In addition, an oscillating electric pulse apparatus is being constructed for the purpose of experimentally verifying the above theory. (3) Affinity-purified anti-ubiquitin and anti-ubiquitin-BSA conjugate from sheep have been prepared and characterized. These antibodies were used to probe endogenous ubiquitin and ubiquitin-protein conjugates in various tissues, and used for immunocytochemical analysis of thin sections of brain. (4) Purification and characterization of ubiquitin activating enzyme from rabbit reticulocytes and human erythrocytes, and of isopeptidase are in progress. (5) Kinetics and mechanism of  $\text{Fe(II)(ferrozine)}_3$  formation from  $\text{Fe(II)(amino acid)}_3$  was investigated. A stepwise mechanism was proposed and rate constants assigned. (6) Model study to examine the theory and practice for determining binding stoichiometry using the Job plot variation method was carried out using the  $\text{Fe(II)-ferrozine}$  system. The results revealed that for cooperative binding systems, anomalous stoichiometries can be obtained.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00203-15 LB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cellular Regulation of Enzyme Levels

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Cynthia N. Oliver	Special Volunteer	LB, NHLBI
	Pamela E. Starke-Reed	Staff Fellow	LB, NHLBI

## COOPERATING UNITS (If any)

Dr. David L. Snyder, Lobund Laboratory, University of Notre Dame, Notre Dame, IN

## LAB/BRANCH

Laboratory of Biochemistry

## SECTION

Section on Enzymes

## INSTITUTE AND LOCATION

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## TOTAL MAN-YEARS:

2.4

## PROFESSIONAL:

2.0

## OTHER:

0.4

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In order to study the fate of specific enzymes in vivo, we have examined the levels of oxidized proteins, specific enzyme inactivation and protease activity in isolated hepatocytes of rats exposed to 100% oxygen for 54 hours and of rats of various ages, 3 to 26 months. Our studies indicate that oxidized proteins accumulate during 48 hours of oxygen treatment. Over the same time interval, at least two enzymes (glucose-6-phosphate dehydrogenase and glutamine synthetase) exhibit a decrease in specific activity without a concomitant loss of immunological cross reactivity. Between 48 and 54 hours of oxygen treatment, the levels of oxidized proteins decrease and the levels of alkaline proteases which exhibit selectivity for the oxidized proteins increase. These results are consistent with the interpretation that oxidized proteins increase in vivo during exposure to high oxygen and are subsequently degraded, possibly by selective proteases which are induced or activated. Similar studies carried out with rats of various ages indicate that oxidized proteins and enzymes are inactivated during normal aging. In this model, however, there is little loss of immunological cross-reactivity; moreover, the level of alkaline proteases which selectively degrade oxidized proteins is lower in old animals compared to young animals.

Similar parameters were examined in rat testis from the Lobund Aging Studies. In these experiments, oxidized proteins were compared in conventionally maintained and in germ-free animals that were either diet-restricted or full-fed.

52



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00203-15 LB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cellular Regulation of Enzyme Levels

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Cynthia N. Oliver	Special Volunteer	LB, NHLBI
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## TOTAL MAN-YEARS:

2.4

## PROFESSIONAL:

2.0

## OTHER:

0.4

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In order to study the fate of specific enzymes in vivo, we have examined the levels of oxidized proteins, specific enzyme inactivation and protease activity in isolated hepatocytes of rats exposed to 100% oxygen for 54 hours and of rats of various ages, 3 to 26 months. Our studies indicate that oxidized proteins accumulate during 48 hours of oxygen treatment. Over the same time interval, at least two enzymes (glucose-6-phosphate dehydrogenase and glutamine synthetase) exhibit a decrease in specific activity without a concomitant loss of immunological cross reactivity. Between 48 and 54 hours of oxygen treatment, the levels of oxidized proteins decrease and the levels of alkaline proteases which exhibit selectivity for the oxidized proteins increase. These results are consistent with the interpretation that oxidized proteins increase in vivo during exposure to high oxygen and are subsequently degraded, possibly by selective proteases which are induced or activated. Similar studies carried out with rats of various ages indicate that oxidized proteins and enzymes are inactivated during normal aging. In this model, however, there is little loss of immunological cross-reactivity; moreover, the level of alkaline proteases which selectively degrade oxidized proteins is lower in old animals compared to young animals.

Similar parameters were examined in rat testis from the Lobund Aging Studies. In these experiments, oxidized proteins were compared in conventionally maintained and in germ-free animals that were either diet-restricted or full-fed.

52



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00204-21 LB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Protein Structure: Enzyme Action and Control

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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Peter A. Cohen Chemist (8/30/87-7/31/88) LB, NHLBI  
Myun K. Han Staff Fellow (5/8/88-) LB, NHLBI  
Francis Cyran FAES Fellow (8/1/88-9/31/88) LB, NHLBI  
Patrick J. Donohue Chemist (8/14/88-) LB, NHLBI

## COOPERATING UNITS (if any)

J.B. Hunt, NSF (Chem. Div.); A. Shrake, Bur. Biologics; H.K. Schachman and  
Edd Eisenstein, Univ. of California, Berkeley; D. Eisenberg, Univ. of California,  
Los Angeles; J.R. Knutsen, Lab. Technical Development, NHLBI

## LAB/BRANCH

Laboratory of Biochemistry

## SECTION

Section on Protein Chemistry

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

4.3

## PROFESSIONAL:

4.0

## OTHER:

0.3

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

(1) Nucleotide analogs have been introduced as structural probes into active sites of dodecameric glutamine synthetase from Escherichia coli. These enzyme derivatives are being used for X-ray structural analysis in D. Eisenberg's laboratory at UCLA and for determining relative intra-subunit distances between the active sites and Trp 57 and Trp 158.

(2) Reversible temperature-induced transitions of glutamine synthetase involve the exposure of 1 of the 2 Trp residues/subunit and 2 of the 17 Tyr residues/subunit without dissociation or aggregation of the dodecameric enzyme. Thermally induced transitions appear to involve the melting of active site structures and at least 2 domains unfold independently.

(3) Thermodynamic studies of Zn(II)-induced stacking of glutamine synthetase dodecamers are in progress.

(4) Regulatory subunits of E. coli aspartate transcarbamoylase bind Zn(II) with high affinity; kinetic and equilibrium constants for this interaction have been determined.

(5) The structure and function of transcriptional factor TFIIIA from oocytes of Xenopus laevis are being investigated with respect to its proposed "Zn(II) finger" structure and specific interactions between TFIIIA and 5S RNA, DNA encoding for 5S RNA, and RNA polymerase III, and possibly other transcriptional protein factors.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00205-33 LB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Occurrence and Biochemical Roles of Selenium in Selenoproteins and Seleno-tRNAs

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Thressa C. Stadtman, Chief, Section on Intermediary Metabolism and Bioenergetics, Laboratory of Biochemistry, NHLBI

Others: Joe Nathan Davis Laboratory Research Assistant LB, NHLBI

## COOPERATING UNITS (if any)

Gas Research Institute, Chicago, Illinois  
Dr. August Böck, University of München, München, West Germany  
Dr. Dolph Hatfield, National Cancer Institute, NIH

## LAB/BRANCH

Laboratory of Biochemistry

## SECTION

Intermediary Metabolism and Bioenergetics

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2.1

## PROFESSIONAL:

1.0

## OTHER:

1.1

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The highly specific occurrence of selenocysteine in several selenium-dependent enzymes is now well established and in at least two cases its co-translational insertion is directed by the opal stop codon, UGA. For this process serine is esterified to a special tRNA having an anticodon complimentary to UGA and then the seryl-tRNA is converted to selenocysteinyl-tRNA which is used for protein synthesis. Selenomethionine, in contrast, is inserted ubiquitously into many proteins in animals, plants, and bacteria as a non-specific substitute for methionine; its specific occurrence has not been documented. Thus, the reported presence of selenocysteine in a Salmonella typhimurium mutant defective in synthesis of specific selenoenzymes (and also seleno-tRNAs) suggested this might be a case of random replacement of cysteine in proteins with selenocysteine. Analysis of this mutant and also a pleiotrophically similar Escherichia coli mutant revealed that both organisms retain the ability to synthesize selenocysteine and selenomethionine and that these selenoamino acids are associated with many proteins of the cells in a form that requires strong acid hydrolysis or proteolytic digestion for liberation. The nature of the mutation that prevents normal highly specific incorporation of selenium into formate dehydrogenases and into tRNAs and instead appears to result in selenocysteine inclusion in many proteins has special significance regarding selenium toxicity and will be investigated in greater detail.

Replacement of the essential selenocysteine residue in a formate dehydrogenase of E. coli with a cysteine residue by site-directed mutagenesis resulted in an enzyme with markedly lower catalytic activity. Procedures have been developed for purification of both forms of the enzyme in order to compare accurately the relative effectiveness of a selenol versus a thiol as a redox center in an enzyme. Both enzymes are extremely oxygen labile and difficult to isolate. The sulfur enzyme is estimated to be 10 or at most 20% as active as the selenium form.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00206-29 LB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Stereochemical Studies of Enzymatic Reactions

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Lin Tsai

Research Chemist

LB, NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Biochemistry

## SECTION

Intermediary Metabolism and Bioenergetics

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.3

## PROFESSIONAL:

1.0

## OTHER:

0.3

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

- (1) The synthesis of 5-carboxymethylaminomethyl-2-selenouracil has been improved.
- (2) Glutamate semialdehyde was tentatively assigned to the carbonyl component that produced the unknown derivative from the hydrolysate of derivatized oxidized glutamine synthetase. This suggests that proline or arginine might be the amino acid that is oxidized in the protein.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00211-15 LB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Role of Protein Oxidation in Protein Turnover and in Aging

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: E. R. Stadtman Chief, Laboratory of Biochemistry LB, NHLBI

Others: B. S. Berlett Biologist LB, NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Biochemistry

## SECTION

Section on Enzymes

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.9

## PROFESSIONAL:

0.6

## OTHER:

1.3

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A number of iron-containing compounds of biological origin were found to replace synthetic iron chelates in catalyzing the peroxidation of amino acids. Detailed studies with a system comprised of hydrogen peroxide, bicarbonate, hemin or ferritin was found to mimic the bicarbonate-dependent oxidation of leucine by the more conventional Fenton reagent (hydrogen peroxide plus Fe(III) plus EDTA). A complete analysis of the reaction products established that leucine oxidation occurs by three independent pathways: (1) Oxidative deamination to form alpha-ketoisocaproic acid and ammonia; (2) Oxidative decarboxylation-deamination to form isovaleraldehyde, bicarbonate and ammonia; (3) Oxidation to the isovaleraldehyde oxime and carbon dioxide. Material and oxidation-reduction balances demonstrate that these three reactions and the amino acid independent iron-catalyzed decomposition of hydrogen peroxide to oxygen account for 90-95% of the hydrogen peroxide and leucine which are consumed. By means of difference spectroscopy, it was shown that there is a time-dependent interaction between Fe(III), oxygen and amino acid. This together with results of earlier studies showing that amino acid oxidation is insensitive to radical scavengers supports the conclusion that amino acid oxidation involves in situ generation activated oxygen species within the Fe(III)- or Fe(II)-amino acid complex, and is not the result of a subsequent free-radical attack of the amino acid by oxygen radicals generated via an amino acid independent mechanism.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00212-17 LB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Glutamine Synthetase in E. coli K12

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Mary Anne Berberich

Research Chemist

LB, NHLBI

## COOPERATING UNITS (if any)

John Smith, Senior Investigator, Seattle Biomedical Research Institute,  
Seattle, Washington

## LAB/BRANCH

Laboratory of Biochemistry

## SECTION

Section on Enzymes

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.3

## PROFESSIONAL:

1.0

## OTHER:

0.3

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Previous studies (Berberich, 1985, J. Bacteriol. 163, 1109) suggested that further metabolism of some effector D-amino acids might result in the generation of a specific "signal" molecule which could elicit the ammonium nitrogen-limited stress response. Although mass spectrometric measurements revealed that the principal recipient of the amino nitrogen of each of these D-amino acids is L-serine probably via increased serine hydroxymethyltransferase activity, SHMT (Berberich and DeMoll, 1988, Abstr., ASM, 228), the conversion of D-glutamate, D-lysine or D-threonine to glycine is not as yet understood.

It has been determined that D-threonine is not a substrate or an inducer of threonine dehydrogenase (TDH), threonine dehydratase, or 2-amino-3-ketobutyrate ligase, enzymes which function in the threonine utilization cycle (TUT) generating glycine and serine (Ravnikar and Somerville, 1987, J. Bacteriol. 169, 2611). That the D-amino acids may be substrates of a specific D-amino acid dehydrogenase which also produces a 2-amino-3-keto acid will be investigated since the D-amino acids are without effect when added to anaerobically cultured cells. It was observed that strains carrying mutations in glyA, the gene for SHMT, are deficient in response to the D-amino acids. When added simultaneously with the D-amino acids, each of the following: L-serine, adenine (but not hypoxanthine), glutamine, azaserine, trimethoprim; eliminates the D-amino acid response indicating that the effective consequence of increased serine synthesis via SHMT may involve the availability of glycine, formyl donors and/or purine regulation. Because about 30% of the spontaneous, second site suppressors of glnD mutations demonstrated high constitutive levels of GS and could be genetically located at glnB (Berberich, 1987, Abstr., ASM, 203), glnB-lacZ fusion strains were constructed. It was determined that in the absence of the glnB gene product, nitrogen control is eliminated. The level of GS in the fusion strains remains repressed upon addition of D-amino acids or upon growth under nitrogen-limited growth conditions. Studies on the regulation of glnB are in progress.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00224-11 LB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Calcium-Regulated Protein Phosphatase

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Charles Y. Huang Research Chemist LB, NHLBI

Others: Aile Zhang Visiting Fellow (Term expired 3/88) LB, NHLBI  
Francesca Santini Visiting Fellow (Term started 2/88) LB, NHLBI  
P. Boon Chock Section Chief LB, NHLBI  
Rixin Zhou NIH Special Volunteer LB, NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Biochemistry

## SECTION

Metabolic Regulation

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2.3

## PROFESSIONAL:

2.1

## OTHER:

0.2

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

(1) In the absence of calmodulin, the mechanism of calcineurin activation by Ni(II) is different from that in the presence of calmodulin. Two Ni(II) ions can bind to the enzyme, one leads to activation, the other to deactivation. However, both the free enzyme and the activated enzyme-Ni(II) complex appear to be thermally less stable than the enzyme-two Ni(II) complex. (2) At least two forms of Ca(II)-inhibited phosphatases from bovine brain have been identified. A 52,000 molecular weight form was found to catalyze only non-protein phosphates. Another form was found to utilize histone H2B as substrate. (3) The intrinsic Ca(II) dissociation constants for the four binding sites of calmodulin have been determined according to the latest model based on x-ray crystallography, NMR, and Ca(II) binding data. The values of the constants are consistent with the existence of two Ca(II)-binding domains: A high-affinity domain with two highly cooperative binding sites and a low-affinity domain with two less cooperative binding sites. When combined with the target enzyme, the low-affinity sites must undergo dramatic conformational change and acquire much higher affinities for Ca(II). (4) Model studies using the Fe(II)-ferrozine system to examine the theory and practice of determination of binding stoichiometry by the continuous variation method (the Job plot) revealed that (a) the system can be best described by a one-step or infinite cooperativity mechanism in which three molecules of ferrozine combine with one Fe(II), and that (b) at low total molar concentration of the two reactants, when the Fe(II) concentration is expressed in terms of binding sites, anomalous stoichiometries may be obtained. The apparent stoichiometry increases with decreasing total molar concentration and varies between 1 (the correct value) and 3.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00225-11 LB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mixed-Function Oxidation of Proteins

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Rodney L. Levine	Senior Investigator	LB, NHLBI
Others:	Michel Chevalier	Visiting Fellow	LB, NHLBI
	Isabel Climent	Visiting Fellow	LB, NHLBI
	John Boutelje	Visiting Fellow	LB, NHLBI
	Miranda Marsh	NIH Special Volunteer	LB, NHLBI

COOPERATING UNITS (if any) Department of Enzymology, Gessellschaft für Strahlen- und Umweltforschung (GSF), Munich, West Germany; Department of Microbial Biochemistry, KabiGen AB, Stockholm, Sweden; Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts

## LAB/BRANCH

Laboratory of Biochemistry

## SECTION

Enzymes

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

5.5

## PROFESSIONAL:

4.5

## OTHER:

1.0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Chronic and acute oxygen toxicity is implicated in a growing list of pathologic processes. The three major biopolymers are targets of oxygen toxicity: Nucleic acids, lipids, and proteins. Many proteins are subject to covalent modification by mixed function oxidation. In general, oxidatively modified enzymes lose catalytic activity and become susceptible to proteolytic degradation. Oxidative modification of proteins may have physiological roles. It appears to be the mechanism by which Klebsiella aerogenes controls the metabolic switch from anaerobic to aerobic metabolism. When these cells are switched to an oxygen atmosphere, a rapid oxidative inactivation provides a coordinated response. Glycerol dehydrogenase, ethanol dehydrogenase, and 1,3 propanediol oxidoreductase are inactivated, but the constitutive dehydrogenases remain active. The oxidative inactivation requires synthesis of RNA and protein. The latter may be an oxidase which generates hydrogen peroxide because the coordinated inactivation is also mediated by hydrogen peroxide without protein synthesis. Compounds which specifically oxidize target enzymes may have therapeutic value. The reverse transcriptase and protease of the human immunodeficiency virus are rational targets for such site-specific oxidations. Experiments with the reverse transcriptase demonstrated that it was susceptible to oxidative inactivation.

Oxidative modification introduces carbonyl groups into the amino acid side chains of the protein. Derivatization of these carbonyl groups with fluoresceinamine provides a sensitive label for oxidized proteins. Hydrolysis of the labelled protein yielded one major labelled amino acid, the fluoresceinamine derivative of γ-glutamyl semialdehyde. Oxidation of amino acid homopolymers demonstrated the generation of γ-glutamyl semialdehyde from arginine, lysine, and proline residues.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00261-03 LB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

CO Dehydrogenase and Acetoclastic Methanogenesis in Methanosarcina barkeri

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: David A. Grahame

Staff Fellow

LB, NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Biochemistry

## SECTION

Intermediary Metabolism and Bioenergetics

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.3

## PROFESSIONAL:

1.0

## OTHER:

0.3

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In experiments with Methanosarcina barkeri it was determined that an intact and energized cell membrane is required for carbon-carbon bond cleavage of acetate as measured by isotopic exchange of carbon dioxide. Although extensive isotopic exchange was not detected in soluble preparations, methanogenesis and methyl-SCoM formation from acetate were readily observed in the presence of ATP and hydrogen. The rate of in vitro methanogenesis was dependent upon both ATP and acetate concentrations. The lag before onset of methanogenesis was not due to a slow production of methyl-SCoM, but rather to activation of step(s) needed for reduction of methyl-SCoM to HSCoM and methane. Both processes of methanogenesis and methyl-SCoM formation from acetate were stimulated by coenzyme A which indicates that acetyl-CoA is the activated form of acetate required for cleavage in methanogenesis. A convenient method was developed for analysis of the enzyme responsible for formation of methyl-SCoM from a methylated corrinoid compound and HSCoM. Cells of M. barkeri grown on acetate exhibited essentially the same level of this enzyme as cells grown on methanol. This suggests a common methyl group transfer step in both pathways of acetate and methanol metabolism. The methyltransferase enzyme was purified to near homogeneity and was found to be monomeric with a molecular weight of 37,000. It showed a high affinity for HSCoM ( $K_m = 0.086$  mM), but weakly bound methylcobalamin ( $K_m = 13.5$  mM). Metal ion chelators inhibited the enzyme but reactivation was brought about by removal of the chelator or addition of divalent metal ions. Supplementation of extracts producing methane and methyl-SCoM from acetate with purified carbon monoxide dehydrogenase (CODH) and the methyltransferase, respectively, failed to stimulate these processes. This suggests that the levels of CODH and methyltransferase were saturating under the conditions tested and that another unidentified step(s) was predominantly rate limiting.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00263-03 LB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Signal Transduction Mechanism Involving Phosphoinositides

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Sue Goo Rhee	Research Chemist	LB, NHLBI
	Pann-Ghill Suh	Visiting Fellow	LB, NHLBI
	Sang Yeol Lee	Visiting Fellow	LB, NHLBI
	Jung Hye Kim	Fogarty Fellow	LB, NHLBI
	Kyung Ho Moon	Special Volunteer	LB, NHLBI
	Hae Won Suh	Special Volunteer	LB, NHLBI
	Sung Ho Ryu	Visiting Fellow	LB, NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Biochemistry

## SECTION

Section on Metabolic Regulation

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

7.1

## PROFESSIONAL:

6.7

## OTHER:

0.4

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

(1) Three phosphoinositide-specific phospholipase C isozymes (PLC-I, II, and III) have been purified from bovine brain. The phospholipase C-related cDNA clones corresponding to PLC-I, II, and III were isolated from rat brain  $\lambda$ gt11 expression cDNA library using specific monoclonal antibodies and sequenced. Each of them encodes a distinct polypeptide with a calculated molecular mass of 138,225 (PLC-I), 148,431 (PLC-II), and 85,840 (PLC-III). Comparison of the sequence of these three isozymes revealed a low overall sequence homology. Nevertheless, a significant amino acid sequence similarity among the three enzymes was found in two regions, one region of about 150 amino acids and the other of about 120 amino acids. The two conserved domains were separated by a variable region. The variable region sequence of PLC-II is relatively long and contains regions homologous to the noncatalytic domain of the nonreceptor protein tyrosine kinases, indicating that PLC-II and cytoplasmic tyrosine kinases might be regulated by a common cellular component. The variable regions of PLC-I and III were short and appeared to be unrelated to these tyrosine kinases. Thus, the modulation of all three enzymes might be quite different.

(2) Two forms of inositol 1,4,5-trisphosphate 3-kinase from bovine brain IPK-1 were partially purified and characterized. IPK-1 was activated about 6-fold by calmodulin in the presence of calcium, whereas IPK-2 is nearly insensitive to CaM.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00265-02 LB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Factors Affecting Expression of a Selenium-Containing Enzyme

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Milton J. Axley Staff Fellow LB, NHLBI

Others: Thressa C. Stadtman Section Chief LB, NHLBI

## COOPERATING UNITS (if any)

Dr. August Böck, University of München, München, West Germany.

## LAB/BRANCH

Laboratory of Biochemistry

## SECTION

Intermediary Metabolism and Bioenergetics

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.4

## PROFESSIONAL:

1.1

## OTHER:

0.3

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The primary step any organism takes in order to sustain life involves controlling gene expression. The bacterium Escherichia coli produces a selenocysteine-containing enzyme, formate dehydrogenase, when grown under anaerobic conditions. We have used this as an easily manipulable model system for analyzing the regulation of gene expression at the transcriptional and translational levels. Fusions of the DNA sequences controlling formate dehydrogenase expression with the gene for an easily detectable enzyme marker, beta-galactosidase, have provided a simple assay system for factors affecting formate dehydrogenase synthesis.

Recent reports have suggested that increased DNA supercoiling mediates the expression of certain anaerobic-specific genes (genes expressed only under anaerobic conditions) in facultatively-anaerobic bacteria. According to this model, expression of bacterial anaerobic-specific genes requires the activity of DNA gyrase, which adds negative supercoils to DNA. In contrast to the results found for other anaerobic-specific genes, it was found that inhibition of gyrase activity enhances the expression of formate dehydrogenase.

Mammals, birds, and several species of bacteria incorporate selenium as selenocysteine at specific sites of a few proteins. The mechanism by which selenocysteine is incorporated into protein remains a mystery. In all known cases, the messenger RNA codon for selenocysteine is UGA, which normally is a stop codon terminating synthesis of the polypeptide chain. Recently, a tRNA was discovered in the laboratory of Dr. August Böck which recognizes a UGA codon and cotranslationally inserts a selenocysteine residue into the growing polypeptide chain. Using this tRNA, an in vitro system for translation of the formate dehydrogenase gene can be constructed, allowing elucidation of the biochemical machinery for selenocysteine incorporation.

113





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00266-02 LB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cloning of Selenoprotein A Gene From *Clostridium sticklandii*

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Gregory E. Garcia Staff Fellow LB, NHLBI

Others: Thressa C. Stadtman Section Chief LB, NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Biochemistry

## SECTION

Intermediary Metabolism and Bioenergetics

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.4

## PROFESSIONAL:

1.1

## OTHER:

0.3

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Selenoprotein A of the glycine reductase complex from *Clostridium sticklandii* has been purified and partially sequenced around the selenocysteine residue. An oligonucleotide probe to this region has been synthesized and shown by Southern blot analysis to hybridize to a single 2300 base pair (bp) fragment of clostridial DNA digested to completion with Hind III.

A clostridial gene library of 1500 to 3000 bp DNA fragments from a complete digestion with Hind III was constructed in pUC-13 and cloned into *Escherichia coli* strain DH5. Fifteen clones were selected by colony hybridization to the probe.

Restriction enzyme digestion analysis of the selected clones revealed that each clone contains the same 2300 bp clostridial DNA fragment. The fragment was digested with restriction enzyme TaqI to yield four smaller fragments. A 400 bp fragment was found to hybridize to the probe. It has been subcloned into the restriction enzyme AccI site of bacteriophage M13 for the production of a single-stranded template for DNA sequencing.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00267-02 LB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Ubiquitination

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	James C. Cook	Staff Fellow	LB, NHLBI
Others:	P. Boon Chock	Chief, Section on Metabolic Regulation	LB, NHLBI
	Luz Hermeda	Special Volunteer	LB, NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Biochemistry

## SECTION

Section on Metabolic Regulation

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.3

## PROFESSIONAL:

1.0

## OTHER:

0.3

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Affinity-purified anti-ubiquitin antibodies from sheep have been developed and characterized, and have been used in a number of applications. These applications include: (1) use as a probe to detect endogenous ubiquitin and ubiquitin-protein conjugates in Western blotting of tissue lysates, (2) use as reagents for immunocytochemical analysis of thin sections of brain and eye lens, and (3) when coupled to a solid support, use as an immunoaffinity chromatography matrix.

Other work has centered on developing a purification scheme for "ubiquitin activating enzyme", E1, from human blood.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00268-02 LB

## PERIOD COVERED

Pctpber 1. 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Oxidation of Proteins and Model Polymers

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J. Michael Poston

Research Chemist

LB, NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Biochemistry

## SECTION

Section on Enzymes

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.3

## PROFESSIONAL:

1.0

## OTHER:

0.3

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Homopolymers of L-alanine, L-arginine, L-histidine, L-lysine, and L-proline and the mixed polymer of L-lysine and L-phenylalanine have been oxidized in the presence of ferrous iron and a chelator, generally citrate. Oxidation introduces carbonyl groups into the polymer which may be converted into secondary amines by reacting them with p-aminobenzoic acid in the presence of sodium cyanoborohydride. Polymers thus derivatized exhibit characteristic spectra which may be measured. The polymers are also cleaved by the oxidation; poly-L-histidine appears to be especially vulnerable to this sort of attack. The greatest rate of oxidation is found when the ratio of ferrous iron to citrate is 1:2.

123



## NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00269-01

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Conformational Analysis of Altered Enzymes Generated by Mixed-Function Oxidation

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Mark T. Fisher

Staff Fellow

LB, NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Biochemistry

## SECTION

Section on Enzymes

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.3

## PROFESSIONAL:

1.0

## OTHER:

0.3

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Glutamine synthetase from *E. coli* is readily inactivated by mixed-function oxidation (MFO) wherein an activated oxygen species is thought to be generated by a protein bound transition metal such as Fe or Cu. Protection of glutamine synthetase against oxidative damage can be afforded by specific substrates (glu, ADP, etc.), metal cofactors (magnesium, manganese), and the transition state analog, L-methionine-SR-sulfoximine phosphate. This evidence strongly suggests that the location of this potential Cu or Fe binding site and activated oxygen generation is at the glutamine synthetase active site. To elucidate the structural origins of both MFO-dependent inactivation reactions and increased proteolytic susceptibility, absorption difference and second derivative rapid-scan spectroscopies were employed to monitor changes in tyrosine and tryptophan microenvironments. Through the use of these spectral probes, the thermodynamic and kinetic alterations of a reversible thermal transition in glutamine synthetase were determined. MFO inactivation of glutamine synthetase results in decreased thermal stability, increased polarity surrounding tyrosine residues, and increased rates of proteolysis. The site-specific nature of the MFO inactivation was further demonstrated by SDS-PAGE analysis of inactivated glutamine synthetase. Extensive exposure (greater than 4 hours) to nonenzymic mixed-function oxidation systems results in highly specific cleaved protein fragments whose quantity depends on the type of metal used (Fe or Cu), source of reducing equivalents (DTT, visible light + flavin, ascorbate) and the oxygen concentration.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00270-01 LB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Oxidative Modification of Biomacromolecule

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: In Kwon Youn Visiting Fellow LB, NHLBI

## COOPERATING UNITS (if any)

Dr. Makoto Matsukura, Clinical Oncology Program, NCI  
Dr. Jack S. Cohen, Clinical Pharmacology Branch, NCI

## LAB/BRANCH

Laboratory of Biochemistry

## SECTION

Section on Enzymes

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.3

## PROFESSIONAL:

1.0

## OTHER:

0.3

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

(1) A highly fluorescent derivative of fluorescein amine was prepared from fluorescein amine to detect the presence of carbonyl groups in oxidatively modified proteins. Glutamine synthetase oxidized by MFO systems reacts with this dye to give a dye-protein conjugate under reductive coupling conditions which can be visualized as fluorescent bands after SDS-PAGE; but this dye does not seem to be applicable to other oxidatively modified proteins.

(2) Several new types of potent inhibitors of HIV were synthesized and tested for inhibitory activity to HIV. (i) Phosphorothioate oligodeoxynucleotide analogs conjugated with EDTA (S-ODN-EDTA) may produce hydroxyl radical in the presence of Fe(II) to cause irreversible damage to the corresponding complementary sequence of RNA. (ii) To increase its affinity for the target sequence, the S-ODN-EDTA derivative was linked with a strong intercalator such as acrylidine. The S-ODN-EDTA conjugate and S-ODN were equally effective in inhibiting the cytopathic effects of HIV-infected T4-cells. Moreover, the EDTA conjugate was less effective in inhibiting gag-protein synthesis in chronically HIV-infected cells.

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## Annual Report of the Laboratory of Cardiac Energetics

National Heart, Lung and Blood Institute  
October 1, 1987 through September 30, 1988

The major goal of the Laboratory of Cardiac Energetics is to develop a better understanding of the cellular processes involved in the conversion of energy to useful work in the heart in vivo. With this new insight we hope to develop new strategies for studying this phenomenon as well as for the prevention and treatment of heart disease.

The cellular metabolic process we have been concentrating on over the last year is the interaction between oxidative phosphorylation and muscle contraction in the heart. Myocardial muscle contraction is believed to occur by utilizing the energy in adenosine triphosphate (ATP) produced predominately by oxidative phosphorylation in the heart. To use this energy, ATP is hydrolyzed to adenosine diphosphate (ADP) and inorganic phosphate (Pi). Previously, it was believed that the rate of oxidative phosphorylation was controlled by the cytosolic feedback of the ATP hydrolysis products, ADP and Pi. To test this basic hypothesis, we have performed experiments on the heart in vivo simultaneously monitoring the cellular contents of ATP, ADP and Pi (using  $^{31}\text{P}$  nuclear magnetic resonance (NMR)), myocardial work and respiration during systematic increases in work output. Myocardial work was varied in these studies using electrical pacing, epinephrine and phenylephrine infusions. The results from these studies demonstrated that the cytosolic concentrations of ATP, ADP and Pi do not change, within the sensitivity of the NMR measurement (10%), during up to 4 fold increases in myocardial oxygen consumption or work. Modeling this data demonstrated that a simple bi-reactant model of respiratory control by ADP and Pi could not explain these results. Thus, either the kinetics of ADP and Pi control of mitochondrial respiration are much more complex than previously believed or other parameters are involved in this process. The other parameters which need to be considered are the redox state and membrane potential of the mitochondria as well as the delivery of oxygen.

In order to monitor these parameters we have been developing techniques for monitoring blood flow and the redox state of mitochondria in vivo. Blood flow is now being monitored on-line using a ultrasonic transit time flow probe modified to work in high magnetic fields. This system will monitor the time course as well as the magnitude of coronary blood flow changes during various perturbations of cardiac work.

Dr. Heineman and Eng are working on methods of monitoring the redox state of mitochondria in vivo. Oxygen measurements will be based on the optical absorbance of myoglobin and cytochrome oxidase. Dr. Heineman has been successful in monitoring these parameters in a perfused working rabbit heart preparation which we will use to develop these optical techniques further. Dr. Eng has developed a single cell NADH fluorescence procedure which has permitted the measurement of the topology of the NADH fluorescence in a single cell. This preparation will be used in the



critical evaluation of the NADH fluorescence signal as an analytical technique in monitoring the mitochondrial NADH signal. If found to be a sound analytical tool, this fluorescence method will be used to evaluate the localized changes in within the cytosol as well as in the intact heart in vivo.

Toward the future, methods are currently under investigation concerning the use of stable isotopes,  $2H$ ,  $13C$  and  $15N$  to follow the metabolism of metabolic substrates as well as other substances in order to evaluate the rate and control of substrate oxidation in the heart. In addition, Dr. Berkowitz has been working on methods of monitoring metabolites using their proton NMR signal. These studies have concentrated on the simplification of the complex in vivo proton spectrum in order to extract quantitative information on specific metabolites such as lactate and polyols.

In a recently completed study by Drs. Portman and Heineman, the developmental aspects of respiratory control in the sheep heart were evaluated using the same techniques outlined above. We performed these studies since preliminary data suggested that the regulatory mechanisms in the newborn animal may be quite different from the adult. Sheep were selected for this study since neonatal and adult animals of the same species could be studied using the techniques we have developed. The adult sheep behaved much like the mature dogs described above. That is, no change in ATP, ADP or  $P_i$  was detected in these hearts with moderate increases in work output. In contrast, the neonatal lamb hearts ADP and  $P_i$  content did change significantly with moderate changes in work. The magnitude of these changes in cytosolic ADP and  $P_i$  are sufficient to explain the changes in respiration observed. These results suggest that ADP and  $P_i$  may play a more prominent role in the regulation of respiration in the newborn heart than in the adult. Thus, a mechanism of respiratory regulation seems to be operational in the adult myocardium which is either not present or relatively inactive in the newborn. These differences in respiratory control through development maybe extremely valuable with regard to identifying the control mechanisms which are operational in the adult heart. With further development of other techniques to monitor oxygen and the mitochondrial redox state, the neonatal heart may also serve as a useful control in the evaluation of the mature hearts regulatory mechanisms.

The Laboratory of Cardiac Energetics was formed during this report period from a group operating within the Laboratory of Kidney and Electrolyte Metabolism (Dr. Maurice Burg, Chief). During this period a productive collaboration on the analysis of organic solutes in mammalian tissues was initiated. Since the establishment of the Laboratory of Cardiac Energetics, this collaboration has continued on several levels involving the regulation and control of organic solutes in mammalian tissues.

In these studies, Dr. Steven Wolff and Dr. Crit Moonen (BEIB) have developed methods of imaging sodium using  $^{23}Na$  NMR in animal models as well as man. These studies have provided a unique insight into the distribution of Na in the renal medulla and the effect of various physiological and pharmacological perturbations. Dr. Wolff has also characterized the  $^{31}P$  NMR spectrum from the in vivo rabbit kidney. He



subsequently used this approach to identify a "new" organic solute in the cortex of the kidney, phosphoethanolamine, and determined the in vivo time course of glycerolphosphorylcholine content during various forms of diuresis in the rabbit kidney in vivo. Using an HPLC method devised by Dr. Wolff and Dr. Yancey (LKEM) which can monitor all of the prevalent organic solutes in kidney extracts, Dr. Wolff is now evaluating which solute or condition, Na, urea or total osmolality and ionic strenght, is a more potent regulator of organic solute content in vivo.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 04601-01 CE

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Control of Cellular Energy Metabolism

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: R. S. Balaban Chief LCE, NHLBI

Others: F. W. Heineman Medical Staff Fellow LCE, NHLBI  
M. A. Portman Guest Researcher NRSA, NHLBI  
J. A. Swain Senior Investigator CSB, NHLBI  
J. Eng Research Scholar HHMI, NHLBI

## COOPERATING UNITS (if any)

Cardiac Surgery Branch and Howard Hughes Medical Institute

## LAB/BRANCH

Laboratory of Cardiac Energetics (formerly under LKEM)

## SECTION

Cardiac Energetics

## INSTITUTE AND LOCATION

National Heart, Lung and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

4.5

## PROFESSIONAL:

3.5

## OTHER:

1

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The control of energy metabolism within the intact heart is being investigated using a variety of techniques and preparations. The purpose of these studies is to determine the mechanisms involved in the interaction of cellular energy conversion, by oxidative phosphorylation or glycolysis, with cardiac muscle contraction and subsequent pumping of blood. These studies are being conducted using a combination of classical techniques as well as state of the art non-invasive methods involving nuclear magnetic resonance (NMR) and optical spectroscopy to monitor the intracellular metabolic effects of changes in cardiac work output in vivo. NMR is used to monitor the cellular concentrations of adenosine di- and tri-phosphates, inorganic phosphate, creatine phosphate and pH. The mitochondrial redox state and oxygenation, in vivo, is being monitored using optical spectroscopy. These experiments have demonstrated that the phosphate metabolites do not change during physiological alterations of steady state work output. Rapid transient studies (resolution <8 sec) also demonstrated that no transient change occurred in these metabolites during a work jump. Changes in the high energy phosphates occurred only when the work performed exceeded the metabolic capacity of the tissue. These data suggest that these phosphate metabolites are to the key cytosolic intermediates in the regulation of myocardial oxidative metabolism as once believed. Optical data suggest that the mitochondrial NADH redox state does change appropriately (i.e. becomes more reduced) with an increase in work. These data suggest that the control of energy conversion in the heart is maybe controlled at the level of substrate oxidation or oxygen delivery to the myocardium.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 04602-01 CE

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Non-Invasive Techniques for Monitoring Cellular Function and Structure

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	R. S. Balaban	Chief	LCE, NHLBI
Others:	B. A. Berkowitz	Staff Fellow	LCE, NHLBI
	M. A. Portman	Guest Researcher	NRSA, NHLBI
	J. Eng	Research Scholar	HHMI, NHLBI
	C. Moonen	Senior Investigator	BEIB, DRS
	S. D. Wolff	Research Scholar	HHMI, NHLBI
	F. W. Heineman	Medical Staff Fellow	LCE, NHLBI
	K. Lim	Research Associate	Stanford University

## COOPERATING UNITS (if any)

Biomedical Engineering and Instrumentation Branch, Howard Hughes Medical Institute and Stanford University

## LAB/BRANCH

Laboratory of Cardiac Energetics

## SECTION

Non-invasive technology

## INSTITUTE AND LOCATION

National Heart, Lung and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

4

## PROFESSIONAL:

4

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

These investigations are devoted to the development of non-invasive methods of accessing cellular structure and function. Two general techniques are being used: Nuclear Magnetic Resonance (NMR) and optical spectroscopy. Over the last year we have developed and demonstrated the following NMR techniques: A two dimensional NMR technique was devised which permits the spectral editing of several metabolites simultaneously in the complicated proton spectrum in vivo. An on line blood flow method capable of use within the powerful magnetic field required for NMR experiments was developed and is now in routine use. A method of monitoring choline metabolism in vivo was developed using the stable NMR isotope deuterium labeled uniformly on the methyl groups of the compound. Using optical spectroscopy, a method of monitoring the topology of mitochondrial fluorescence in a single cardiac myocyte was developed as well as a method of monitoring and imaging the NADH fluorescence of the surface layers of the heart in vivo.



# ANNUAL REPORT OF THE CARDIOLOGY BRANCH

National Heart, Lung, and Blood Institute

October 1, 1987 through September 30, 1988

The experimental interests of the Cardiology Branch focus 1) on elucidating the cellular mechanisms responsible for the development of microvascular angina (MVA) and hypertension, including the possible roles of endothelial dysfunction and of aberrations of cytosolic calcium handling; 2) examining the potential roles of growth factors, the adrenergic nervous system, and the cellular regulation of calcium in the pathogenesis of hypertrophic cardiomyopathy (HCM); 3) determining the mechanisms responsible for dynamic alterations of coronary vascular resistance in MVA and peripheral vascular resistance in hypertension; 4) developing new techniques for opening coronary obstructions.

## DYSFUNCTION OF THE CORONARY MICROVASCULATURE AS A CAUSE OF MYOCARDIAL ISCHEMIA

The syndrome of angina pectoris occurring in the absence of coronary artery disease or vasospasm of the large coronary arteries has been a diagnostic dilemma. Over the past 4 years we have explored the concept that this common clinical disorder is due to dysfunction of the small intramural coronary arteries.

Microvascular coronary dysfunction and angina: We previously demonstrated that about 70% of patients with angina-like pain but normal large coronary arteries had inadequate increases in coronary flow and decreases in coronary resistance in response to pacing-induced stress, abnormalities exacerbated by ergonovine. The chest pain was associated with diminished myocardial lactate consumption and other findings consistent with myocardial ischemia. LV dysfunction developed in these pts during exercise. We also demonstrated reduced coronary vasodilator reserve in response not only to metabolic stimuli (pacing-induced increase in  $\text{MVO}_2$ ) but also to dipyridamole, a potent pharmacologic dilator of coronary arterioles. Further analyses of these data suggested that the flow limitation is due to narrowing of the small pre-arteriolar coronary arteries, rather than of the arterioles, per se. On the basis of these findings, we now refer to this syndrome as microvascular angina (MVA).

Evidence of a diffuse disorder of smooth muscle: Several other intriguing findings evolved from these original studies. Abnormal esophageal motility was found in over half of the pts with MVA, suggesting this syndrome is characterized by a general increase in smooth muscle tone. This concept was further substantiated by examining the peak reactive hyperemic response of the forearm to ischemia. Peak flow was reduced and vascular resistance was higher after 5 minutes of forearm ischemia in pts with MVA compared to an age and sex matched control group. In addition, because dyspnea is common in these pts and seemed disproportionate to the severity of myocardial ischemia, we determined whether there also is dysfunction of bronchial smooth muscle causing airway obstruction, and therefore contributing to the dyspnea experienced by MVA pts. We measured  $\text{FEV}_1$  in normal controls and in pts with MVA before and after inhalation of incremental doses of methylcholine. Methylcholine caused a 20%



decrease in FEV<sub>1</sub> from baseline in the majority of MVA pts, a response observed uncommonly in control subjects. Thus, dyspnea in MVA pts may be due to hyper-reactivity of bronchial smooth muscle. More importantly, these results provide further substantiation of the hypothesis that the syndrome of MVA involves a generalized disorder of vascular and nonvascular smooth muscle function.

Aberrations of cytosolic calcium levels as a cause of MVA: We demonstrated last year that the voltage-dependent calcium channel blockers, verapamil, nifedipine and diltiazem, improve symptoms in most pts with MVA. Some pts, however, were unresponsive to these agents. Lidoflazine, a calcium antagonist that blocks calcium entry in sites other than the voltage-dependent channel, improved effort tolerance and chest pain in these pts. Moreover, the drug improved abnormally low coronary flow and high coronary resistance present during pacing and after dipyridamole. These results suggest two important conclusions. First, that the elevated coronary resistance in MVA is not totally fixed but is due, at least in part, to elevated vasoconstrictor tone reversible by certain vasodilator drugs and second, that these vasoconstrictor influences may be mediated by elevated cytosolic calcium levels, such that the basic abnormality and resulting symptoms are improved by calcium entry blocking drugs.

#### CHANGES IN CORONARY VASCULAR RESISTANCE AS A CAUSE OF VARIATION IN ISCHEMIC EVENTS IN CORONARY ARTERY DISEASE

Pts with coronary artery disease (CAD) exhibit a circadian variation in frequency of ischemic episodes and the apparent ease with which ischemia is precipitated (ischemic threshold). To define the mechanisms of these phenomena, we measured ischemic threshold and minimal forearm vascular resistance (obtained following 5 min of forearm ischemia) in CAD pts at 8:00, 13:00, 17:00 and 22:00 hours. Ischemic threshold was defined as the heart rate at onset of 1 mm ST segment decrease during exercise. Changes in minimal (post-ischemic) forearm vascular resistance (VR) were taken as indices of changes in coronary VR present during exercise-induced ischemia. Ischemic threshold and minimal VR varied at different times of the day. There was a strong association between minimal VR and ischemic threshold, such that in each pt at the time of day when VR was highest, ischemic threshold was lowest, and vice versa. There was a simultaneous and inverse circadian variation in both ischemic threshold and minimal VR. Ischemic threshold was lower and VR higher in the morning and at night compared to noon and evening. Thus, CAD pts have a circadian variation in ischemic threshold. Our results suggest this may be caused by an inverse variation in coronary VR, as reflected by the relation we found between minimal forearm VR and ischemic threshold. We therefore believe that circadian variations in coronary VR probably account for circadian variations experienced by CAD pts in 1) the frequency of transient ischemic episodes, 2) the ease with which increases in  $\dot{MVO}_2$  can precipitate ischemia, 3) exercise capacity, 4) the onset of AMI, sudden cardiac death, and other cardiac events.





## PATHOPHYSIOLOGIC MECHANISMS CONTRIBUTING TO HYPERTENSION AND ANGINA IN HYPERTENSIVE HEART DISEASE

Impaired endothelium-dependent vascular relaxation as a contributing cause of hypertension: Endothelium regulates vascular tone by secreting substances that modulate the activity of surrounding vascular smooth muscle. Acute and chronic hypertension impairs endothelium-mediated vasodilation in experimental animals. To determine whether pts with hypertension have an endothelium-dependent abnormality in vascular relaxation, we studied the vascular response of hypertensive pts and normal subjects to acetylcholine, an endothelium-dependent vasodilator, and to nitroprusside, a direct smooth muscle dilator. Drugs were infused into the brachial artery, and the forearm vascular responses were measured. We found that the vasodilator response to nitroprusside was unaltered in hypertensive pts but that to acetylcholine was attenuated. These results indicate that endothelium-mediated vasodilatation is impaired in pts with hypertension, a defect that may either exacerbate or causally contribute to the hypertensive process. The finding also may account for the heretofore unexplained observation that in some pts with hypertension and angina, coronary vascular resistance paradoxically increases in response to the increased flow produced by the metabolic stress of pacing. Normally, the increased shear stress caused by increased flow leads to an endothelial-mediated vasodilatation. However, if endothelium-mediated vasodilatation is impaired, as it appears to be in hypertension, the increased shear stress could actually increase coronary vascular resistance, resulting in a reduction in coronary flow and thereby predispose to myocardial ischemia and angina.

Microvascular dysfunction of the small coronary arteries as a cause of angina in pts with hypertension: Pts with hypertension frequently have angina-like chest pain, despite the presence of normal epicardial vessels. Because the systemic arteries of hypertensive pts are constricted, we reasoned that a similar abnormality might involve the small coronary arteries. We therefore evaluated hypertensive pts with angina but without CAD by measuring coronary vasomotor responses to rapid atrial pacing before and after the administration of intravenous ergonovine. With atrial pacing the increase in coronary flow was less in hypertensive pts with angina compared to normotensive control subjects. Ergonovine caused coronary vasoconstriction in the hypertension pts, but not in the normals. Thus, hypertensive pts with angina but without CAD may have myocardial ischemia due to elevated coronary microvascular resistance that can be exacerbated by vasoconstrictor stimuli.

### STUDIES ON CELLULAR MECHANISMS RESPONSIBLE FOR THE PATHOPHYSIOLOGIC DEVELOPMENT OF HYPERTROPHIC CARDIOMYOPATHY

We are testing several hypotheses regarding the pathophysiologic processes responsible for the development of hypertrophic cardiomyopathy (HCM): 1) There is an abnormality in cellular regulation of cytosolic calcium; 2) There is an abnormality in cardiac norepinephrine (NE) kinetics; 3) There is a proliferative disorder present.

Abnormalities in Cardiac Calcium Regulation. Because the hearts of HCM pts are hyperdynamic and exhibit impaired relaxation, and because these abnormalities and pts' symptoms respond to calcium antagonists, we examined the hypothesis that



abnormal calcium fluxes may be present in HCM. To test this concept we measured the density of dihydropyridine binding sites present in the hearts of HCM pts undergoing surgery, and found that the density of these binding sites (and presumably therefore the density of voltage sensitive calcium channels), is elevated in HCM pts, whereas beta adrenergic receptor density is unchanged. These results suggest that a specific increase in the voltage sensitive calcium channel plays an important primary role in the pathophysiology of HCM.

Abnormalities in Cardiac Norepinephrine Kinetics: It has long been proposed that abnormal NE kinetics may be present in HCM, mainly because these pts exhibit a hyperdynamic LV and because their symptoms respond to beta blocking agents. We therefore examined cardiac NE kinetics and found that the production rate of NE (arteriovenous difference across the heart times coronary blood flow) was elevated in HCM. The increased spillover of NE into the blood stream was not due to increased secretion by the nerve terminal, but to impaired neuronal uptake of NE. It is likely that this defect increases NE levels at myocardial and vascular smooth muscle adrenoreceptors, and thereby contributes to the pathophysiology of HCM, including myocardial hypertrophy, hyperdynamic LV, hyperplasia of the media of small coronary arteries, and myocardial ischemia due to vasoconstriction of the small coronary arteries.

Evidence Favoring a Proliferative Disorder: Pathologic examination of myocardium from HCM pts reveals myocardial hypertrophy, excess of scar tissue, hyperplasia of the smooth muscle present in small coronary arteries, and possible angiogenesis. These findings are compatible with the hypothesis that HCM may be a primary proliferative disorder. We are presently in the process of undertaking several studies to examine the validity of this hypothesis.

Acidic and basic fibroblast growth factors (aFGF, bFGF) are angiogenic peptides and mitogens for fibroblast, smooth muscle, and endothelial cells. They have been identified in many tissues of mesenchymal origin, but not in the heart. This year we demonstrated that aFGF and bFGF are present in both normal human heart and in hearts from pts with HCM. Thus, we found in 4 normal human hearts that fractions eluted from heparin sepharose columns at 1 and 1.5 M NaCl were mitogenic for 3T3 fibroblasts and for endothelial cells. By Western blotting we found the 1.0M fraction cross-reacted with polyclonal antisera raised against peptide replicates of aFGF (15,000 MW) and the 1.5 M fraction cross-reacted with polyclonal antisera for bFGF (18,000 MW). The cellular origin of these peptides was determined by homogenizing freshly isolated cardiac myocytes and employing heparin affinity chromatography.. By Western blotting with specific antisera for aFGF and bFGF, we identified these peptides in the 1.0 M and 3.0 M fractions eluted from the heparin-sepharose columns. Thus, aFGF and bFGF are present in abundance in cardiac myocytes. Most recently we used heparin-affinity chromatography to determine whether these factors are also present in myocardium obtained at the time of LV myectomy in pts with HCM. The column fractions eluted at 1.0 - 2.0 M NaCl had potent mitogenic activity, stimulating DNA synthesis 2 to 6-fold. The mitogenicity of these fractions was potentiated up to 2-fold by the addition of heparin, a characteristic of aFGF. Thus, myocardium derived from pts with HCM contains



potent mitogens, most likely aFGF and bFGF. These data, along with the considerations discussed above, raise the intriguing hypothesis that HCM may be caused by increased production of, or increased sensitivity to, these growth factors.

Because of the presence of aFGF and bFGF in HCM hearts, and the proposed abnormality in calcium regulation, we determined whether the mitogenic responses to FGF included smooth muscle cells and, if so, whether the response was associated with increases in cytosolic calcium. Cultures of rat aortic smooth muscle cells were chronically exposed to bFGF. Cytosolic calcium was measured (by fura 2) 12 hrs after 4 days of exposure to bFGF. In confluent cultures grown in 1 or 5% fetal bovine serum, bFGF accelerated cell proliferation and induced a dose-dependent sustained increase in intracellular calcium concentration. Thus, these results suggest a possible interrelationship between abnormalities in the production of, or response to, FGF and abnormalities in intracellular calcium regulation.

### ANGIOGENESIS IN ISCHEMIC MYOCARDIUM

Oncologic research has determined that increases in certain solid tumor cell populations must be preceded by an increase in new capillaries that converge upon the tumor and supply it with blood. This hypothesis implies that angiogenesis is a rate-limiting step to most solid neoplasms. It also led to studies seeking to identify those factors responsible for neovascularization (and therefore tumor expansion), with the ultimate hope of developing substances that would inhibit angiogenesis (and thus tumor growth). We were intrigued by the thought that we might pursue an analogous but opposite approach; to use angiogenic factors to promote rather than to inhibit blood vessel growth in ischemic myocardium. We have therefore initiated studies to determine whether it is possible to potentiate angiogenesis in ischemic myocardium. Our studies involve 2 parallel lines of investigation.

The cellular biology of angiogenic factors in myocardium. As previously noted, we demonstrated that aFGF and bFGF is present in normal human heart. Last year we examined a rat model of myocardial ischemia, by removing the hearts of rats and incubating them at 24°C for 60 min. Using a polyclonal antiserum against bFGF, we found that ischemia increased bFGF levels and that the mitogenic effect of tissue extracts on 3T3 fibroblasts was also significantly higher in the ischemic hearts. Because it seemed unlikely that bFGF was newly synthesized and released by ischemic myocardial cells, we hypothesized that myocardial acidosis released pre-existing stores of bFGF from the extracellular matrix and thereby made it more bio-available. Further studies substantiated this hypothesis, suggesting that myocardial acidosis occurring during ischemia stimulates the release of bFGF. We have recently extended these studies to test the effects of hypoxia on mitogenic factors released from cultured endothelial cells. Confluent LE-II cells, derived from mouse lung capillary endothelium, were exposed to 5% O<sub>2</sub> for 3 hr/day for 4 days. The conditioned medium and cell lysates were analyzed by heparin-affinity chromatography. The 0.5 M and 2.0 M salt eluates of the hypoxic cell lysate and conditioned media were more mitogenic than that of control cells. Mitogenicity of the 1.1 M eluate was not increased by hypoxia. These data indicate that hypoxia stimulates capillary endothelial cells to produce and



release PDG and bFGF. We therefore conclude that myocardial ischemia may initiate collateral formation by increasing the bio-availability of several growth factors which, by diffusing out of the ischemic area into surrounding normal myocardium, stimulates the genesis and ingrowth of coronary collaterals. Compatible with this concept are the results of our histologic studies, which demonstrated that cell proliferation in response to myocardial infarction occurs in the border zone of ischemia, almost exclusively in capillary and venular endothelial cells and fibroblasts. The proliferative response extends up to 3 mm into noninflamed, nonischemic tissue.

Stimulation of collateral growth in vivo: There are many CAD pts who develop refractory ischemic symptoms and are not candidates for initial or repeat bypass surgery. We are thus assessing the potential of angiogenic substances to stimulate collateral growth in a dog model of chronic ischemia. The model we have devised is based on the observation that intracoronary collaterals develop following implantation of the internal mammary artery (IMA) into ischemic regions of the LV between the IMA and the native coronary vessels (Vineberg operation), but that the flow capacity of these collaterals is not adequate to importantly influence clinical outcome. We are currently implanting IMA grafts into the anterior LV wall of dogs; the dogs are randomly assigned to receive continuous administration into the IMA of potential angiogenic substances or normal saline. The area of LV into which the IMA graft is placed is rendered ischemic over a 2-4 week period by applying an ameroid constrictor around the LAD coronary artery. Animals are studied 8 wks post-operatively to determine ischemic myocardial flow at baseline and during maximal vasodilator stimulation. We are also studying the effects of IMA occlusion on LV function. Our first study demonstrated that implantation of the IMA provided nutritional flow in 50% of dogs; however, the magnitude of flow was not significantly different from control, indicating that implantation of an IMA, at least in this dog model of ischemia, is not an effective means of myocardial revascularization. Thus, it is an excellent model to determine the angiogenic potential of test agents.

We have studied the efficacy of heparin alone in promoting angiogenesis. Although heparin caused a dose-related increase in collateral flow, the increase was not significant. We are currently evaluating the effects of heparin and aFGF on collateral flow in this ischemic model.

#### CATHETER-BASED TECHNIQUES TO OPEN OCCLUDED CORONARY ARTERIES

For the past 5 years, a multidisciplinary research group coordinated through NHLBI and the Cardiology Branch, has investigated the feasibility of new technologies (laser angioplasty, thermal angioplasty, mechanical atherectomy devices, endovascular prosthetic devices, and angioscopy), in an attempt to expand the range of patients with coronary and peripheral vascular disease who would be candidates for percutaneous intravascular remodeling procedures.

Laser tissue interaction studies have been performed on human necropsy tissues to determine the histologic and thermodynamic effects of varying laser sources. Superficial tissue ablation, without associated thermal tissue injury, can be optimized with a combination of proper wavelength selection,





(ultraviolet or infrared) and lasing parameters (pulsed versus continuous). We believe that Excimer lasers (at 308 nm) and developmental Erbium:YAG lasers (at 2940 nm) may provide the most advantageous tissue responses if appropriate fiber optic delivery catheters can be developed. To compliment these basic laser-tissue interaction studies, extensive work has been done in the area of feedback control systems to improve the specificity and safety of laser tissue ablation. Laser-induced fluorescence spectroscopy has been studied as a technique to differentiate atheroma from normal tissue and underlying normal media. Almost 60 patients have been studied in vivo and sensitive algorithms have been established which can differentiate atheroma from normal tissue. Based upon these data, clinical studies have been initiated utilizing a dual laser system, incorporating features of fluorescence plaque recognition with pulsed dye laser (480 nm) tissue ablation. This system is unique in that pulse laser firing is determined by a feedback signal which indicates that the proposed target site is indeed atheromatous material. Thus far, 10 patients with peripheral vascular disease have been treated utilizing this "probe and treat" laser angioplasty concept. Successful laser recanalization was achieved in 8 of 10 chronic total occlusions in which standard guidewire techniques were unsuccessful. We propose to utilize a similar device for percutaneous applications in patients with chronic total occlusions of the coronary arteries.

Significant further work has been accomplished to determine the particle size number and distribution associated with mechanical rotational atherectomy catheters. These devices hold great promise for definitive removal of large volume atheroma and we will be initiating a study to examine the effects of such a device to improve revascularization of unfavorable coronary vessels during the course of coronary artery bypass graft surgery.

#### STUDIES EXAMING THE PROBLEM OF SUDDEN DEATH IN HCM

Because sudden death occurs frequently in HCM pts, it is necessary to determine its mechanism so that more effective therapy can be designed. We first compared the long-term outcome of HCM pts who survived a cardiac arrest (CA) to that of HCM pts without ventricular tachycardia (VT) and HCM pts with VT on 24-hr Holter monitoring. Operation was performed in most pts with CA and LV outflow obstruction to relieve the large gradients present. We found that pts with VT had a worse prognosis than pts without VT, and that pts resuscitated from CA had an even worse prognosis. Further, the worst prognosis was found in those pts resuscitated from CA who had the nonobstructive form of HCM. Of CA pts who had electrophysiologic study, the large majority had both AV node and/or His/Purkinje conduction abnormalities, as well as inducible sustained VT. In another investigation we performed EP studies in HCM pts with syncope, presyncope, and/or VT on Holter. We found that VT could be induced in a high percentage of pts 1) who had prior CA, 2) who had syncope or presyncope even in the absence of VT, and 3) who had no presyncope or syncope but who had VT on Holter. Thus, EP studies in HCM pts suggest that symptoms of impaired consciousness and Holter evidence of VT are markers of ventricular electrical instability.



In an attempt to determine whether the induction of VT in the EP lab can be predicted by noninvasive study, we employed signal averaging ECG to detect late potentials. We found that signal averaging is a useful noninvasive test to predict HCM pts with inducible VT. We believe such studies may help determine pts at risk of sudden death and who therefore are candidates for more aggressive antiarrhythmic therapy, as can be achieved by an implantable defibrillator.

#### CORONARY ARTERY DISEASE

Silent ischemia on Holter monitoring in asymptomatic and mildly symptomatic pts with CAD: Many investigators are currently recommending that all pts with CAD must have Holter monitoring studies at frequent intervals to ascertain the frequency of silent ischemic events so as to develop an adequate therapeutic strategy regarding medical and/or surgical therapy. The implications of this recommendation are profound, in that it will substantially increase the cost of medical care delivery. It was our belief that standard treadmill stress testing might provide all of the necessary clinical information and thereby make frequent Holter monitor recordings unnecessary. We therefore characterized the frequency of daily myocardial ischemia as recorded by Holter monitoring and related it to ischemic threshold determined by treadmill stress testing in pts with CAD who were asymptomatic or had only mild symptoms. Ischemic threshold was determined as the time of exercise at onset of 1 mm ST segment depression. We found that about half of CAD pts with no or mild symptoms have ischemic episodes during daily life, which are often silent. However, treadmill stress testing provided virtually all of the important prognostic information. Pts with a negative stress test usually do not exhibit ischemic episodes on Holter monitoring. In pts with positive stress tests, the magnitude of daily myocardial ischemic episodes is predicted by the ischemic threshold -- pts who develop ischemia at low levels of ischemic threshold have frequent episodes of ischemia on Holter monitoring, whereas pts with a high ischemic threshold have no or rare such episodes. Hence, routine treadmill stress testing predicts the findings on Holter monitoring and therefore, at this time, Holter monitoring provides little if any additional prognostic information.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 HL 04146-02 CB
PERIOD COVERED October 1, 1987 to September 30, 1988		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Smooth muscle growth and lamin-rich matrix		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
Yi Fu Zhou, M.D.	Guest Researcher	CB NHLBI
Fernando Bazoberry, M.D.	Special Volunteer	CB NHLBI
Victor Ferrans, M.D.	Chief, Ultrastructure Section	PB NHLBI
Ward Casscells, M.D.	Senior Investigator	CB NHLBI
COOPERATING UNITS (if any)  Pathology Branch, NHLBI		
LAB/BRANCH Cardiology Branch		
SECTION Experimental Physiology and Pharmacology		
INSTITUTE AND LOCATION NHLBI, NIH, Bethesda, MD		
TOTAL MAN-YEARS: 0.5	PROFESSIONAL: 0.4	OTHER: 0.1
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>             In addition to providing a structural support, the extracellular matrix (ECM) exerts important influences on cellular proliferation, migration and differentiation, and affects such processes as embryogenesis, wound healing, regeneration and angiogenesis. A convenient preparation is the gel extract of the EHS tumor, which is biochemically and structurally similar to an endothelial type of basement membrane. This gel promotes differentiation in endothelial cells, hepatocytes, Sertoli cells and Schwann cells. We examined its effect on smooth muscle cells because of the problem that these cells de-differentiate in culture and lose their contractile properties. We found that subconfluent rat aortic smooth muscle cells proliferated faster on the EHS gel. Their growth was also less inhibited when serum was withdrawn than was the case for cells on plastic alone. This suggested that ECM components might be storing growth factors produced by the cells or found in serum. The individual matrix components, laminin, or heparin were unable to reproduce the effects of the gel. Electron microscopy indicated that cells grown in EHS gel produced less of their own ECM. Also, the cells grown in the gel were grouped in small bundles. Basement membrane components may thus promote growth by enhancing cell to cell contact and subsequent paracrine functions. However, when confluent quiescent cells were refed, the EHS gel inhibited growth. (Heparin, but not laminin, also inhibited growth). However, the electron microscopic appearance remained that of a non-contractile cell, and there was no increase in intracellular calcium in response to depolarization by potassium chloride. For this reason we will not be pursuing this project further, but it is clear that basement membrane components influence smooth muscle cell growth in vitro, and in vivo, may influence such properties as atherosclerosis, embryogenesis and the response to arterial wounds, such as those produced by angioplasty.           </p>		

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 04151-02 CB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Purification of Myocardial Heparin-Binding Growth Factors

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Ward Casscells, M.D.	Senior Investigator	CB	NHLBI
Edith Speir, B.S.	Biochemist	CB	NHLBI
Lena Haggroth, B.S.	Guest Researcher	CB	NHLBI
Michio Chiba, M.D.	Guest Researcher	CB	NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Cardiology Branch

## SECTION

Experimental Physiology and Pharmacology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

2

## PROFESSIONAL:

1.9

## OTHER:

0.1

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A year ago we isolated from diseased and normal human left ventricular myocardium, factors of molecular weight 14,000 to 18,000, which were mitogenic for fibroblast and which could be identified on western blotting with some antisera to acidic and basic fibroblast growth factors. This year we found that these growth activities are destroyed by heat, acid, or tripsin, blocked by anti-FGF antisera and by protamine (which blocks the FGF receptors). They also stimulated capillary endothelial cell migration and displaced bFGF in a radioreceptor assay. We confirmed the immunological similarities to aFGF and bFGF with several more antisera and also proved the specificity of these by showing that these bands were no longer produced on Western blotting after the antiserum had absorbed to excess recombinant aFGF or bFGF respectively. Some immuno-reactivity was also seen at Mr 35,000, which may represent the FGF precursor.

These growth factors were also present in right atrial tissue (but not in blood) and were present in a sample of fresh nonischemic normal heart as well, indicating that they are not due to rapid synthesis in response to agonal ischemia.

FGF peptides may normally have morphogenic and neurotropic functions but we are currently investigating their potential roles in pathologic processes such as ischemia and hypertrophy.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 04174-02-CB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Promotion of angiogenesis by heparin and non-anticoagulant heparin

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Ellis F. Unger, M.D.	Senior Staff Fellow	CB	NHLBI
Cedric D. Sheffield, M.D.	Staff Fellow	CB	NHLBI
S. Ward Casscells, M.D.	Senior Staff Fellow	CB	NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB	NHLBI

## COOPERATING UNITS (if any)

Veterinary Resources Branch, NIH

## LAB/BRANCH

Cardiology Branch

## SECTION

Experimental Physiology and Pharmacology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland

## TOTAL MAN-YEARS:

1.2

## PROFESSIONAL:

0.7

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Both heparin and non-anticoagulant heparin fragments are known to potentiate angiogenesis in vitro. The purpose of this investigation is to test the ability of heparin and non-anticoagulant heparin fragments to promote intercoronary collaterals in a canine model of myocardial ischemia.

In 24 dogs, the origin of the left anterior descending coronary artery (LAD) will be isolated and an ameroid device will be applied to its proximal portion, effecting gradual occlusion over a 2 to 3 week period. Dogs will be randomly assigned to three treatment arms: group 1 will receive heparin; group 2 will receive an equal quantity of non-anticoagulant heparin fragments; and group 3 will receive only 0.9% saline and serve as the control group. Maximal collateral blood flow to the LAD area will be quantitated with microsphere injections during adenosine induced vasodilatation at various times after surgery, so that the time course and maximal extent of collateral growth can be determined and compared in the three groups.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 HL 04175-02-CB
PERIOD COVERED                      October 1, 1987 to September 30, 1988		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <u>In vivo</u> use of endothelial cell growth factor to effect myocardial angiogenesis		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
Ellis F. Unger, M.D.	Senior Staff Fellow	CB    NHLBI
Cedric D. Sheffield, M.D.	Staff Fellow	CB    NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB    NHLBI
COOPERATING UNITS (if any) <p style="text-align: center;">Veterinary Resources Branch, NIH</p>		
LAB/BRANCH                      Cardiology Branch		
SECTION                              Experimental Physiology and Pharmacology		
INSTITUTE AND LOCATION        NHLBI, NIH, Bethesda, Maryland		
TOTAL MAN-YEARS:              2.2	PROFESSIONAL:                  1.2	OTHER:                          1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>During the last several years, great advances have been made in our understanding of angiogenesis, the study of blood vessel growth and proliferation. Several of the agents which promote this process have been well characterized. One promising agent, endothelial cell growth factor (ECGF), has been synthesized through recombinant techniques.</p> <p>The purpose of this investigation is to utilize ECGF in a canine model to effect myocardial angiogenesis, and to direct this process to ameliorate myocardial ischemia. In our model, the left anterior descending coronary artery (LAD) of dogs is occluded gradually over a 2 to 3 week period by an ameroid device applied to the proximal vessel. The internal mammary artery (IMA) is implanted into the region normally supplied by the LAD. It is known that collateral vessels develop from the IMA and other sources to supply the territory normally perfused by the LAD. Myocardial blood flow is generally sufficient under rest conditions, but is inadequate under conditions of stress. We have developed a means to quantitate the maximal conductance between the IMA and the LAD vascular bed, assessing myocardial perfusion, the physiologic result of angiogenesis.</p> <p>Ameroids will be applied to the LAD of 24 dogs. The IMA will be implanted in the LAD area. As heparin is known to stabilize ECGF and promote its effect, dogs will be randomly assigned to receive infusions of ECGF in heparin, heparin alone, or normal saline. After 8 weeks, resting and maximal myocardial blood flow will be quantitated using radiolabeled microspheres. The maximal IMA to LAD bed conductance will be calculated, and comparisons made between groups. In addition, the effect of IMA occlusion on regional left ventricular function will be assessed using sonomicrometry. Vessels will be examined morphometrically and various hematologic, biochemical, and immunologic parameters will be assessed in the 3 groups, to determine potential adverse effects of ECGF.</p>		

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 04177-01 CB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Establishment of a cell line derived from human hypertrophic myocardium

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Pamela Karasik, M.D.	Medical Staff Fellow	Cardiology	NHLBI
Ward Casscells, M.D.	Senior Staff Investigator	Cardiology	NHLBI
Zhou Yi Fu, M.D.	Visiting Scientist	Cardiology	NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	Cardiology	NHLBI

## COOPERATING UNITS (if any)

Surgery Branch, NHLBI

## LAB/BRANCH

Cardiology

## SECTION

Laboratory of Experimental Physiology and Pharmacology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland

## TOTAL MAN-YEARS:

1.5

## PROFESSIONAL:

1.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Hypertrophic cardiomyopathy is a not uncommon form of heart disease, of unknown origin. We are seeking to establish a cell line derived from heart tissue removed from patients with hypertrophic cardiomyopathy at the time of surgery to help determine the cellular cause of this disease.

A. Sterile tissue is obtained from the operating room after left ventricular myotomy and myomectomy. The tissue is manually dispersed and incubated at 37° Celsius, 10% CO<sub>2</sub>, in enriched medium. When a cell layer has grown up, the cells are passaged into flasks, and the culture medium adjusted as needed. The cells seem to grow very slowly, with doubling times of several weeks. They also have unusual morphology and it is not clear at this time if they are fibroblast or smooth muscle in origin.

B. Once the cell line is established and further characterized, we will study its response to growth factors such as fibroblast growth factor and platelet-derived growth factor. We will also examine the intrinsic production of these growth factors by the cardiac cells.

The etiology of hypertrophic cardiomyopathy remains obscure. On a basic level, understanding the function of the individual cellular components may lead to a clearer understanding of the disease as a whole. This work should ultimately complement clinical work being done in the same area.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 04178-01 CB

## PERIOD COVERED

October 1, 1987 - September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Significance of mitral valve prolapse in hypertrophic cardiomyopathy

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Julio A. Panza, M.D.

Guest Researcher

CB

NHLBI

Barry J. Maron, M.D.

Senior Investigator

CB

NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Cardiology Branch

## SECTION

Echocardiography Laboratory

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

0.5

## PROFESSIONAL:

0.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Prominent systolic prolapse of the mitral valve (MVP) is not a common finding in patients with hypertrophic cardiomyopathy. To determine the significance of echocardiographic evidence of MVP in these patients, we studied 22 patients with HCM who also had clear evidence of a large, redundant mitral valve, prolapsing into the left atrium in systole.

The results of this study show that this combination is not associated with an increased risk of complications and, therefore, appears to be part of the wide morphologic spectrum of HCM.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 04179-01 CB

## PERIOD COVERED

October 1, 1987 - September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Silent ischemia in mildly symptomatic coronary artery disease

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Julio A. Panza, M.D.	Guest Researcher	CB	NHLBI
Arshed A. Quyyumi, M.D.	Senior Investigator	CB	NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB	NHLBI
Timothy S. Callahan	Project Coordinator	CB	NHLBI

## COOPERATING UNITS (If any)

None

## LAB/BRANCH

Cardiology Branch

## SECTION

Cardiovascular diagnosis section

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

1

## PROFESSIONAL:

0.6

## OTHER:

0.4

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

It is not clear whether patients with coronary artery disease (CAD) and no or only mild symptoms have episodes of silent ischemia and whether these are related to exercise test parameters. Therefore, we studied 40 patients with 48-hour Holter monitoring and exercise treadmill test.

This study indicates that approximately half of the patients with CAD and no or only mild symptoms have episodes of silent ischemia during daily life. The presence and magnitude of the ischemia episodes is related to exercise parameters of ischemia threshold and, therefore, can be predicted by assessment of the exercise test.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 04180-01 CB

## PERIOD COVERED

October 1, 1987 - September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Non-invasive assessment of dynamic obstruction with continuous-wave Doppler

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Julio A. Panza, M.D.	Guest Researcher	CB	NHLBI
Barry J. Maron, M.D.	Senior Investigator	CB	NHLBI
Ruth Petrone	Echocardiographic Tech.	CB	NHLBI
Yvonne Wesley	Echocardiographic Tech.	CB	NHLBI
Lameh Fananapazir, M.D.	Senior Staff Fellow	CB	NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Cardiology Branch

## SECTION

Echocardiography Laboratory

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

0.5

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Patients with hypertrophic cardiomyopathy (HCM) may present intraventricular gradients as expressions of dynamic subaortic obstruction. A non-invasive technology to measure the gradient is highly desirable for repetitive assessment of the presence and magnitude of obstruction. To determine the utility of continuous wave Doppler (CW) in assessing the left ventricular outflow tract gradient in patients with HCM, we performed simultaneous measurements of CW and pressure gradients.

The results of this study support the use of CW Doppler as a reliable technique to measure the intraventricular gradients in hypertrophic cardiomyopathy. However, in patients with severe obstruction, a particularly narrow outflow tract and a high velocity jet of mitral regurgitation may result in misinterpretation of the magnitude of subaortic obstruction.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 04181-01 CB

## PERIOD COVERED

October 1, 1987 - September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Assessment of microvascular endothelium - dependent reactivity

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Julio A. Panza, M.D.	Guest Researcher	CB	NHLBI
Arshed A. Quyyumi, M.D.	Senior Investigator	CB	NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB	NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Cardiology Branch

## SECTION

Experimental physiology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

1

## PROFESSIONAL:

0.5

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Previous studies have shown that the endothelium regulates vascular tone by modulating the activity of vascular smooth muscle. It has also been demonstrated that the endothelium plays an important role in atherosclerosis and animal models of hypertension.

The present study was designed to determine whether patients with essential hypertension have an endothelium-dependent abnormality in vascular relaxation. For this purpose, we have utilized intra-arterial infusion of acetylcholine (an endothelium-dependent vasodilator) and sodium nitroprusside (a direct smooth muscle dilator) in hypertensive patients and in a control group of normal volunteers.

The results of this study suggest that patients with essential hypertension have an impairment in endothelial-mediated vascular relaxation that may, either cause or exacerbate the hypertensive process.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 04182-01 CB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Natural History of Asymptomatic Patients with Aortic Regurgitation

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Robert O. Bonow, M.D.	Senior Investigator	CB	NHLBI
Edward Lakatos, Ph.D.	Statistician	EC BR	NHLBI
Barry J. Maron, M.D.	Senior Investigator	CB	NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB	NHLBI

## COOPERATING UNITS (if any)

Biostatistics Research Branch, NHLBI

## LAB/BRANCH

Cardiology Branch

## SECTION

Nuclear Cardiology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

1

## PROFESSIONAL:

1

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In a previous report, we demonstrated that asymptomatic patients with chronic severe aortic regurgitation (AR) and normal left ventricular (LV) systolic function have an excellent prognosis, with less than 4% per year requiring aortic valve replacement because symptoms, LV dysfunction, or both develop. In addition, baseline echocardiographic and radionuclide angiographic measurements were useful in identifying a higher risk subgroup. In that report, we included 79 patients with a mean follow-up period of 4 years, during which 12 patients required operation. We have extended this series to 104 patients followed over a mean 8 year period (range 2-15 years), with 25 events (2 patients died suddenly and 23 underwent operation). At 11 years, 58% of patients remained asymptomatic with normal LV function, an attrition rate less than 4%/year. Many variables describing LV function were associated with outcome, but by multivariate life-table analysis, only age, echocardiographic LV systolic dimension at initial study, and the rate of change in LV systolic dimension and in radionuclide angiographic ejection fraction at rest during serial studies were independently associated with outcome. Thus, within subgroups of patients with chronic AR identified at low or high risk by baseline measurements, further stratification according to risk may be performed on the basis of serial changes in LV systolic function with time. In addition, the LV ejection fraction response to exercise is not helpful to predicting outcome in AR, once age and resting LV function are accounted for.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 04183-01 CB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Relation of ECG abnormalities to evolving left ventricular hypertrophy in HCM

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Julio A. Panza, M.D.

Guest Researcher

CB

NHLBI

Barry J. Maron, M.D.

Senior Investigator

CB

NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Cardiology Branch

## SECTION

Echocardiography Laboratory

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

0.3

## PROFESSIONAL:

0.3

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Patients with hypertrophic cardiomyopathy often show substantial increase in left ventricular wall thickness or even de novo development of hypertrophy during childhood. To determine the utility of the electrocardiogram in reflecting such changes in left ventricular mass, serial 12-lead electrocardiograms and two-dimensional echocardiograms were analyzed in 38 children with hypertrophic cardiomyopathy.

The results of this study show that children with hypertrophic cardiomyopathy and evolving left ventricular hypertrophy showed little change in the electrocardiogram during the period of observation. However, electrocardiographic abnormalities may be the initial manifestation of hypertrophic cardiomyopathy, appearing even before left ventricular hypertrophy is detectable by echocardiography, thus providing advance evidence of morphologic changes that will subsequently occur.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 04184-01 CB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Fluorescence-guided laser angioplasty in patients

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Martin B. Leon, M.D.	Senior Investigator	CB	NHLBI
Yaron Almagor, M.D.	Research Fellow	CB	NHLBI
Antonio L. Bartorelli, M.D.	Research Fellow	CB	NHLBI
Louis G. Prevosti, M.D.	Research Fellow	CB	NHLBI
Richard Chang, M.D.	Staff Radiologist	DR	CC
Donald L. Miller, M.D.	Staff Radiologist	DR	CC
Paul D. Smith, Ph.D.	Physical Scientist	BEIB	DRS
Robert F. Bonner, Ph.D.	Senior Physicist	BEIB	DRS

## COOPERATING UNITS (if any)

Diagnostic Radiology, Clinical Center

Biomedical Engineering and Instrumentation Branch, Division of Research Services

## LAB/BRANCH

Cardiology Branch

## SECTION

Cardiovascular Diagnosis Section

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

0.3

## PROFESSIONAL:

0.3

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A prototype dual laser system employing fluorescence spectroscopy for plaque recognition combined with a pulsed dye laser for tissue ablation was utilized to perform fluorescence guided, laser assisted balloon angioplasty in patients with femoro-popliteal occlusions. In 10 patients, we demonstrated successful real-time fluorescence-guided pulsed dye laser ablation of obstructions but noted difficulty with both laser and subsequent balloon angioplasty of heavily calcified lesions. This early clinical experience will permit us to modify delivery systems such that larger laser angioplasty channels may be created with increased catheter tip control.

In addition, we have begun preliminary studies in patients with chronic total coronary occlusions, using a similar fluorescence guided laser system. This will be the first attempt at percutaneous laser recanalization of chronic total coronary occlusions in humans and results are forthcoming.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 04185-01 CB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Coronary plaque recognition by fluorescence spectroscopy

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Martin B. Leon, M.D.	Senior Investigator	CB	NHLBI
Antonio L. Bartorelli, M.D.	Research Fellow	CB	NHLBI
Yaron Almagor, M.D.	Research Fellow	CB	NHLBI
Louis G. Prevosti, M.D.	Research Fellow	CB	NHLBI
Julie A. Swain, M.D.	Senior Surgeon	SB	NHLBI
Charles L. McIntosh, M.D.	Senior Surgeon	SB	NHLBI
Paul D. Smith, Ph.D.	Physical Scientist	BEIB	DRS
Robert F. Bonner, Ph.D.	Senior Physicist	BEIB	DRS

## COOPERATING UNITS (if any)

Surgery Branch, NHLBI

Biomedical Engineering and Instrumentation Branch, Division of Research Services

## LAB/BRANCH

Cardiology Branch

## SECTION

Cardiovascular Diagnosis Section

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

0.1

## PROFESSIONAL:

0.1

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This study represents a continuation of previous work wherein laser-induced fluorescence spectroscopy has been used in the operating room to differentiate normal endovascular tissue sites from atheroma. A low-power Helium Cadmium (325 nm) laser was utilized for short exposures to obtain fluorescence spectra from exposed aorta and coronary tissue in 20 additional patients. Using advanced algorithms for atheroma detection, the specificity and sensitivity for atheroma recognition was 100% and 91% respectively. We concluded that in vivo fluorescence spectroscopy of coronary arteries and aorta discriminates atheroma from normal tissue with high specificity and sensitivity and can be utilized for real-time guidance during laser angioplasty.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 04186-01 CB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Particulate debris from rotational atherectomy

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Martin B. Leon, M.D.	Senior Investigator	CB	NHLBI
Louis G. Prevosti, M.D.	Research Fellow	CB	NHLBI
John A. Cook, Ph.D.	Senior Research Fellow	CB	NCI
Ellis F. Unger, M.D.	Staff Associate	CB	NHLBI
Cedric D. Sheffield, M.D.	Research Fellow	CB	NHLBI
Yaron Almagor, M.D.	Research Fellow	CB	NHLBI
Antonio L. Bartorelli, M.D.	Research Fellow	CB	NHLBI

## COOPERATING UNITS (if any)

National Cancer Institute

## LAB/BRANCH

Cardiology Branch

## SECTION

Cardiovascular Diagnosis Section

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

0.1

## PROFESSIONAL:

0.1

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Rotational atherectomy catheters can ablate atheroma and restore patency in atherosclerotic arteries but little is known about the resultant particulate debris or its effect on myocardial blood flow. Therefore, we examined particulate debris number and size distribution after ablation of human necropsy aorta utilizing a sophisticated laser fluorescence and light scattering particle counter. Almost 90% of the particles were less than 12 microns in size and 80% were less than 5 microns in size. To determine the acute in vivo effects of particle debris on myocardial blood flow, carefully graded injections of particulate were infused into the left circumflex coronary artery of dogs and blood flow was measured using radio-labeled microspheres. Preliminary results indicate that only very large doses of calcified particulate caused deleterious hemodynamic effects. These studies will be continued to formalize our understanding of the safe particle burden which could be tolerated by the coronary microcirculation without significant effects on myocardial blood flow and/or left ventricular hemodynamics.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 04187-01 CB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Arterial vasospasm during thermal angioplasty

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Martin B. Leon, M.D.	Senior Investigator	CB	NHLBI
Louis G. Prevosti, M.D.	Research Fellow	CB	NHLBI
David Y. Lu, M.D.	Research Fellow	CB	NHLBI
Antonio L. Bartorelli, M.D.	Research Fellow	CB	NHLBI
Yaron Almagor, M.D.	Research Fellow	CB	NHLBI
Robert F. Bonner, Ph.D.	Senior Physicist	BEIB	DRS

## COOPERATING UNITS (if any)

Biomedical Engineering and Instrumentation Branch, Division of Research Services

## LAB/BRANCH

Cardiology Branch

## SECTION

Cardiovascular Diagnosis Section

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

0.1

## PROFESSIONAL:

0.1

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Although thermal angioplasty is being used in patients, the biological responses to varying catheter-tip temperatures in small arteries is unknown. Therefore, we studied a canine model consisting of autologous fat, Gelfoam implants into small femoral arteries, followed by prototype catalytic thermal-tip catheter angioplasty. We found that irreversible vasospasm frequently occurs during thermal angioplasty, especially at higher catheter-tip temperatures with prolonged catheter-tissue contact and in smaller vessels which are closely matched to the catheter size, particularly in low flow states, such as severe stenoses, or total occlusions.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 04188-01 CB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Stent implantation in valved conduits

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Martin B. Leon, M.D.	Senior Investigator	CB	NHLBI
Yaron Almagor, M.D.	Research Fellow	CB	NHLBI
Louis G. Prevosti, M.D.	Research Fellow	CB	NHLBI
Antonio L. Bartorelli, M.D.	Research Fellow	CB	NHLBI
Michael Jones, M.D.	Senior Attending Surgeon	SB	NHLBI

## COOPERATING UNITS (if any)

Surgery Branch, NHLBI

## LAB/BRANCH

Cardiology Branch

## SECTION

Cardiovascular Diagnosis Section

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

0.1

## PROFESSIONAL:

0.1

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Although valved conduits have been used successfully in severe forms of right ventricular-pulmonary artery discontinuity, progressive valved conduit stenosis is an important clinical problem. Therefore, we employed a balloon expandable stent in a baboon model consisting of pulmonary artery ligation and implantation of an RV-PA 14 mm bioprosthetic dacron valved conduit. We were able to deploy the balloon expandable stent within the stenotic portion of the valved conduit and in most animals achieved significant reduction in RV systolic pressure and valved conduit gradient. Therefore, we believe the balloon expandable stent may be a useful technique to prolong the patency of right heart valved conduits in patients.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 04189-01 CB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Stent implantation in vein grafts

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Martin B. Leon, M.D.	Senior Investigator	CB	NHLBI
Louis G. Prevosti, M.D.	Research Fellow	CB	NHLBI
Renu Virmani, M.D.	Pathologist	PB	AFIP
Antonio L. Bartorelli, M.D.	Research Fellow	CB	NHLBI
Yaron Almagor, M.D.	Research Fellow	CB	NHLBI
Paul S. Tierstein, M.D.	Research Fellow	CB	NHLBI

## COOPERATING UNITS (if any)

Pathology Branch, Armed Forces Institute of Pathology

## LAB/BRANCH

Cardiology Branch

## SECTION

Cardiovascular Diagnosis Section

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

0.3

## PROFESSIONAL:

0.3

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

Intravascular balloon expandable stents may reduce restenosis of saphenous vein grafts after PTCA. To determine the acute and chronic in vivo effects of a slotted tubular steel stent on vein grafts we deployed 14 stents on balloon catheters in 14 femoral interposition vein grafts in 11 sheep. After allowing the vein grafts to arterialize over approximately 8 months, all stents were deployed and host responses were studied by angiography, light and scanning electron microscopy. At 3 weeks, 8 weeks and 5 months, we found no gross or histologic effects of intimal hyperplasia, vein graft erosion, late thrombosis, or progression of aneurysmal changes. In vein grafts with minimal residual stenosis and in animals given antiplatelet therapy, the patency was excellent (100%) and no deleterious host responses were noted. Thus, treatment of vein grafts with implantable stents is feasible, safe, and may improve long-term PTCA results.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 04190-01 CB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Serial systolic and diastolic LV function in doxorubicin cardiomyopathy

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

B. William Choi, M.D.	Guest Researcher	CB	NHLBI
Kevin McCarthy, M.D.	Senior Staff Medical Officer	NM	NHLBI
Beate Scheffknecht, M.D.	Guest Researcher	CB	NHLBI
Stephen Bacharach, Ph.D.	Medical Physicist	NM	NHLBI
Robert O. Bonow, M.D.	Senior Investigator	CB	NHLBI

## COOPERATING UNITS (if any)

Nuclear Medicine

## LAB/BRANCH

Cardiology Branch

## SECTION

Nuclear Cardiology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

0.3

## PROFESSIONAL:

0.3

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Serial studies on doxorubicin induced cardiomyopathy were studied in relation to systolic function as well as diastolic filling characteristics during rest and exercise.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 04191-01 CB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cardiac performance during exercise in patients with aortic stenosis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

B. William Choi, M.D.	Guest Researcher	CB	NHLBI
Deborah Barbour, M.D.	Senior Staff Fellow	CB	NHLBI
Martin Leon, M.D.	Senior Investigator	CB	NHLBI
Stephen Bacharach, Ph.D.	Medical Physicist	NM	NHLBI
Kevin McCarthy, M.D.	Senior Staff Medical Officer	NM	NHLBI
Robert O. Bonow, M.D.	Senior Investigator	CB	NHLBI

## COOPERATING UNITS (if any)

Nuclear Medicine

## LAB/BRANCH

Cardiology Branch

## SECTION

Nuclear Cardiology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

0.3

## PROFESSIONAL:

0.3

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Exercise-induced symptoms in aortic stenosis were characterized in relation to hemodynamic changes.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 04192-01 CB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

LV systolic function and diastolic filling characteristics in mitral stenosis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

B. William Choi, M.D.	Guest Researcher	CB	NHLBI
Deborah J. Barbour, M.D.	Senior Staff Fellow	CB	NHLBI
Martin B. Leon, M.D.	Senior Investigator	CB	NHLBI
Kevin E. McCarthy, M.D.	Senior Staff Medical Officer	NM	NHLBI
Robert O. Bonow, M.D.	Senior Investigator	CB	NHLBI

## COOPERATING UNITS (if any)

Nuclear Medicine

## LAB/BRANCH

Cardiology Branch

## SECTION

Nuclear Cardiology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

0.5

## PROFESSIONAL:

0.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Systolic dysfunction in mitral stenosis was correlated to diastolic filling characteristics. Exercise induced left ventricular diastolic volume, stroke volume and cardiac output changes in mitral stenosis were compared to normal volunteers.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 04193-01 CB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Programmed stimulation findings in patients with hypertrophic cardiomyopathy

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

Lameh Fananapazir, M.D.

Senior Staff Fellow

CB

NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Cardiology Branch

## SECTION

Clinical Electrophysiology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

0.6

## PROFESSIONAL:

0.6

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The purpose of the present investigation was to determine the types of ventricular arrhythmias induced during programmed electrical stimulation (PES) and the relation of induced ventricular tachycardia to incidence of spontaneous arrhythmia and presenting symptoms in 95 patients with hypertrophic cardiomyopathy (HCM). Sustained VT was induced in 38 (40%) of patients. This consisted of rapid polymorphic VT in 60%, relatively slower monomorphic VT in 32% and ventricular fibrillation in 8% of patients. There was correlation between inducibility of VT and severity of presenting symptoms (sudden cardiac death > syncope > presyncope > asymptomatic) and occurrence of VT on Holter monitoring. These findings suggest that PES truly identifies patients with cardiac electrical instability and severity of symptoms and presence of spontaneous VT may both be markers of this condition.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 04194-01 CB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Correlation of signal averaging with programmed stimulation in HCM patients

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Lameh Fananapazir, M.D.	Senior Staff Fellow	CB	NHLBI
Richard O. Cannon, M.D.	Senior Investigator	CB	NHLBI
Martin B. Leon, M.D.	Senior Investigator	CB	NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB	NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Cardiology Branch

## SECTION

Clinical Electrophysiology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

0.6

## PROFESSIONAL:

0.6

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Signal averaged electrocardiographic (SAE) detection of late potentials (LP) is a non-invasive method for detection of patients with aubrythmogenic ventricle. The purpose of this study was to determine the correlation between SAE and inducibility of sustained ventricular tachycardia invasively by programmed stimulation (PS) in 30 patients with hypertrophic cardiomyopathy. The results show that based on certain LP indices SAE had a sensitivity and specificity of 85% and 94% respectively for induced VT.

We conclude that SAE is a useful non-invasive test which in addition to PS may help to identify HCM patients with electrically unstable ventricles who may be at risk of sudden cardiac death.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 04195-01 CB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Circadian variation in ischemic threshold in coronary artery disease

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Arshed A. Quyyumi, M.D.	Senior Investigator	CB	NHLBI
Julio Panza, M.D.	Guest Researcher	CB	NHLBI
Frederic L. Sax, M.D.	Senior Staff Fellow	CB	NHLBI
Richard O. Cannon, III, M.D.	Senior Investigator	CB	NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB	NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Cardiology Branch

## SECTION

Cardiovascular diagnosis section

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

0.75

## PROFESSIONAL:

0.75

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

There is a circadian variation in the frequency of ischemic episodes in patients with coronary artery disease. The heart rate threshold before the onset of ischemia in these episodes also varies during the day. To define the mechanisms responsible for these variations, we measured ischemic threshold during treadmill exercise tests and minimum forearm vascular resistance, as an index of minimum coronary vascular resistance, at different times of the day in patients with coronary artery disease. Ischemic threshold was defined as the heart rate at the onset of ST segment depression during exercise and minimum forearm vascular resistance was measured after 5 minutes of ischemia.

In 15 patients studied, there was a strong inverse correlation between the minimum forearm vascular resistance and ischemic threshold, so that at the time of day when forearm vascular resistance was lowest, ischemic threshold was highest and vice versa. Furthermore, in the group as a whole, ischemic threshold was lower and vascular resistance higher in the morning and night compared to noon and evening. Thus, patients with coronary artery disease have a circadian variation in ischemic threshold which may be caused by an inverse circadian variation in coronary vascular resistance. These variations may account for the circadian variations in transient ischemic episodes and their variable threshold in patients with coronary artery disease. The mechanisms responsible for circadian variation in vascular resistance need to be further investigated.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 04196-01 CB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Prediction of adverse effects of amiodarone by signal averaging in HCM patients

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Lameh Fananapazir, M.D.

Senior Staff Fellow

CB

NHLBI

Stephen E. Epstein, M.D.

Chief, Cardiology Branch

CB

NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Cardiology Branch

## SECTION

Clinical Electrophysiology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

0.6

## PROFESSIONAL:

0.6

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Analysis of the terminal portion of the QRS of the ECG and detection of late potential (LP) by signal averaging (SAE) is a noninvasive method for identifying patients who are prone to develop ventricular tachycardia (VT) and are therefore at risk for sudden cardiac death. We have earlier shown that there was a good correlation between the presence of LP and inducibility of VT in patients with hypertrophic cardiomyopathy (HCM). Amiodarone is a potent antiarrhythmic drug. The purpose of the present investigation was to determine whether changes in SAE predicted efficacy and proarrhythmic potential in 14 HCM patients treated with amiodarone. Amiodarone increased the incidence of LP. This was associated with the greater ease with which VT was induced during programmed electrical stimulation. Thus SAE may identify HCM patients in whom amiodarone is proarrhythmic and may be at increased risk for sudden cardiac death.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 HL 04197-01 CB
PERIOD COVERED October 1, 1987 to September 30, 1988		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Prognosis and electrophysiologic findings in HCM sudden cardiac death survivors		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)  <div style="display: flex; justify-content: space-between;"> <span>Lameh Fananapazir, M.D.</span> <span>Senior Staff Fellow</span> <span>CB</span> <span>NHLBI</span> </div>		
COOPERATING UNITS (if any)  None		
LAB/BRANCH Cardiology Branch		
SECTION Clinical Electrophysiology		
INSTITUTE AND LOCATION NHLBI, NIH, Bethesda, MD		
TOTAL MAN-YEARS: 0.6	PROFESSIONAL: 0.6	OTHER:
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <div> <input checked="" type="checkbox"/> (a) Human subjects  <input type="checkbox"/> (a1) Minors  <input type="checkbox"/> (a2) Interviews         </div> <div> <input type="checkbox"/> (b) Human tissues         </div> <div> <input type="checkbox"/> (c) Neither         </div> </div>		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>             Identification of hypertrophic cardiomyopathy (HCM) patients who require aggressive antiarrhythmic therapy (e.g., implantation of defibrillator device) requires knowledge of the prognosis of various subgroups of this heterogenous disease. We compared the long-term outcome in 41 consecutive HCM survivors of sudden cardiac death (SCD) with 66 HCM patients without ventricular tachycardia (VT) and 17 patients with VT on 24-hour Holter monitoring. Of the SCD patients, 23 had surgery for left ventricular outflow obstruction (OBST) and 11 patients had electrophysiologic studies (EPS). Five-year event-free (further SCD or death) rates for patients with no VT, with VT, SCD (surgical) and SCD (nonsurgical) were 90%, 76%, 67% and 37% respectively. Of the SCD who had EPS, 8 (73%) had atrioventricular conduction abnormalities and 9 (82%) patients had inducible sustained malignant VT. Thus, (a) SCD conveys a relatively poor prognosis especially in patients who were not surgical candidates, and (b) most SCD patients with HCM have both conduction abnormalities and inducible VT.           </p>		

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 04198-01

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Induction of malignant ventricular tachycardia on amiodarone in HCM patients

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

Lameh Fananapazir, M.D.

Senior Staff Fellow

CB

NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Cardiology Branch

## SECTION

Clinical Electrophysiology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

0.6

## PROFESSIONAL:

0.6

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Although amiodarone has been reported to improve survival in at-risk patients with hypertrophic cardiomyopathy (HCM), we have noted an unexpected mortality rate in highly symptomatic HCM patients treated with this potent antiarrhythmic medication. The study reports the results of programmed electrical stimulation (PS) in 14 HCM patients - 10 patients presented with syncope and 4 with presyncope. All patients had nonsustained ventricular tachycardia (VT) on Holter monitoring. Before amiodarone therapy, sustained VT was induced in 11 patients. In 10 patients the VT was polymorphic. On amiodarone, sustained VT was induced with significantly less aggressive PS protocol in all 14 patients. In 12 patients this was monomorphic VT. Although the VT was slower on amiodarone, in all patients induction of VT was associated with marked hypertension requiring rapid termination. We conclude that in most HCM patients amiodarone facilitates induction of a hemodynamically unstable sustained monomorphic VT and thus its use is potentially dangerous and requires evaluation by PS.

200





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 04199-01 CB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Coronary flow and operative result in hypertrophic cardiomyopathy

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Richard O. Cannon, M.D.	Senior Investigator	CB	NHLBI
Charles L. McIntosh, M.D.	Senior Surgeon	SB	NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB	NHLBI

## COOPERATING UNITS (if any)

Heart Surgery Branch, NHLBI

## LAB/BRANCH

Cardiology Branch

## SECTION

Cardiovascular Diagnosis Section

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

1

## PROFESSIONAL:

1

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Operative relief of left ventricular outflow obstruction improves symptoms in most, but not all, patients with hypertrophic cardiomyopathy. To investigate whether pre-operative measurements of coronary flow reserve predict efficacy, twenty patients underwent study of coronary flow and lactate and oxygen metabolism within the myocardium, in the basal state and during rapid atrial pacing to a heart rate of 150. The six patients with flows  $>175$  ml/min had significantly greater basal left ventricular outflow obstruction ( $90 \pm 30$  vs.  $50 \pm 32$  mmHg,  $p < .01$ ) compared to the remaining 14 patients. Severity of ischemia was similar in the two groups with 5 of 6 of the high flow patients and 8 of 14 of the remaining patients producing lactate during pacing stress. During post-operative study with relief of outflow obstruction, pacing stress resulted in substantially lower flows ( $216 \pm 18$  pre-op, to  $151 \pm 46$  ml/min post-op,  $p < .05$ ) in the 6 pre-op high flow patients compared to a lesser reduction in flow in the other 14 patients ( $138 \pm 19$  preop to  $123 \pm 43$  ml/min postop, NS). Further, there was a substantial improvement in lactate metabolism in these 6 patients ( $28.6 \pm 23.9$  mM x ml/min postoperative, versus  $-32.8 \pm 30.5$  mM x ml/min preoperatively,  $p < .05$ ) with no patient producing lactate. In contrast, there was no net change overall in lactate metabolism in the Group B patients. Further, all 6 high flow patients had a 10 beat/min or greater improvement in pacing angina threshold, whereas 5 of the 14 low flow patients had no improvement.

Thus, a higher peak flow response to stress consistently predicts post-operative improvement to pacing stress. Results in patients with lower peak flow responses are variable, suggesting mechanisms other than obstruction (small vessel disease, myocardial fibrosis, abnormal diastolic function, which might contribute to symptoms and be less likely to be benefited by relief of left ventricular outflow obstruction.

202



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 04200-01 CB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Effect of surgery on ventricular function in hypertrophic cardiomyopathy

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Richard O. Cannon, M.D.	Senior Investigator	CB	NHLBI
Terri F. Rumble, R.N.	Technician	CB	NHLBI
Charles L. McIntosh, M.D.	Senior Surgeon	SB	NHLBI
Robert O. Bonow, M.D.	Senior Investigator	CB	NHLBI

## COOPERATING UNITS (if any)

Heart Surgery Branch, NHLBI

## LAB/BRANCH

Cardiology Branch

## SECTION

Cardiovascular Diagnosis

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

1

## PROFESSIONAL:

1

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In order to study the impact of operation on left ventricular systolic function in hypertrophic cardiomyopathy (HCM), 8 patients underwent simultaneous measurement of left ventricular pressure, and volume during diagnostic study prior to operation, with repeat study approximately 6 months after myotomy-myectomy. Septal myotomy-myectomy reduced the left ventricular outflow gradient from  $75 \pm 61$  to  $18 \pm 30$  mmHg (mean  $\pm 1$  SD,  $p < .01$ ). There was no significant change in the heart rate, blood pressure, stroke volume index, or left ventricular end diastolic volume in the post-operative study compared to the pre-operative study. In 4 patients (Group A) the end systolic pressure/volume relationship, an index of contractility, was shifted to the right in the post-op study compared to the pre-op study, indicating a decrease in contractility. In the other 4 patients (Group B) there was no alteration in this relationship in the post-op study compared to the preoperative study, indicating no change in contractility. In comparing the two groups, the Group A patients had a higher index of contractility (end systolic pressure divided by end systolic volume =  $13.0 \pm 9.9$ ) compared to the Group B patients ( $3.8 \pm 1.4$  mmHg/ml). Following operation there was a substantial reduction in this relationship in the Group A patients (change from pre-op to post-op  $8.6 \pm 7.9$  compared to  $-1.2 \pm 2.5$  mmHg/ml ( $p < .05$ ) in the Group B patients. Despite the pre-op ejection fractions being similar in the two groups, there was a substantial fall in the ejection fraction in the Group A patients ( $17 \pm 5$ ) compared to essentially no change in the ejection fraction of the Group B patients ( $2 \pm 1$ ,  $p < .05$  versus Group A). Thus, myotomy-myectomy appears to reduce contractility in some patients with HCM, especially those with elevated contractility pre-op, and is reflected by a substantial fall in ejection fraction. This may be advantageous by further reducing myocardial oxygen demands, but also potentially deleterious with respect to long-term left ventricular systolic function.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 04801-01 CB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Abnormal bronchial airway function in patients with microvascular angina

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Richard O. Cannon, M.D.	Senior Investigator	CB	NHLBI
David E. Peden, M.D.	Medical Staff Fellow	LCI	NIAIAD
Carol E. Berkebile, R.N.	Staff Registered Nurse	LCI	NIAIAD
Michael A. Kaliner, M.D.	Chief, Allergy Branch	LCI	NIAIAD
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB	NHLBI

## COOPERATING UNITS (if any)

Allergy Branch, NIAIAD

## LAB/BRANCH

Cardiology Branch

## SECTION

Cardiovascular Diagnosis Section

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1

## PROFESSIONAL:

1

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have previously demonstrated that the vasodilator reserve of the coronary microcirculation is reduced after ergonovine in patients with chest pain despite normal coronary angiograms in the absence of epicardial spasm, and term this disorder microvascular angina. We subsequently found limited flow response to ischemia in forearm vessels and abnormal esophageal motility, suggesting a widespread disorder of smooth muscle tone.

Because dyspnea is common in these patients and seems disproportionate to the severity of myocardial ischemia, we studied airway flow ( $FEV_1$ ) to determine whether bronchial smooth muscle is also abnormal in microvascular angina.  $FEV_1$  was measured in 11 patients with microvascular angina before and after incremental doses of methacholine and compared to normal controls. Methacholine caused a 20% decrease in  $FEV_1$  from baseline in 8 of 11 patients at a methacholine dose of  $81 \pm 71$  cumulative units. In contrast, only 2 of 12 control patients had a 20% decrease in  $FEV_1$  at a methacholine dose of  $179 \pm 36$  cumulative units ( $p < .001$ ) a response rate significantly less than patients with microvascular angina ( $p < .02$ ).

Thus, dyspnea in microvascular angina patients may be due to hyperactivity of bronchial smooth muscle. These results further indicate a more generalized disorder of vascular and nonvascular smooth muscle function in this syndrome.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 04802-01 CB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Limitation in peak coronary flow-velocity in microvascular angina

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Richard O. Cannon, M.D.	Senior Investigator	CB	NHLBI
Arshed A. Quyyumi, M.D.	Senior Investigator	CB	NHLBI
William H. Schenke, B.S.	Technician	CB	NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Cardiology Branch

## SECTION

Cardiovascular Diagnosis Section

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

1

## PROFESSIONAL:

1

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We previously demonstrated limitation in great cardiac vein flow response to rapid atrial pacing following ergonovine administration in the absence of epicardial coronary artery disease or spasm, a syndrome we now call microvascular angina. To further study the effect of ergonovine on coronary flow reserve, we measured peak to basal flow velocity with papaverine 15 mg intracoronary, using a steerable Doppler catheter in the proximal left anterior descending artery in 5 patients with angina and angiographically normal coronary arteries. Although the initial peak to basal flow velocity response to papaverine 15 mg was normal ( $4.1 \pm 1.9$ , mean  $\pm$  1 S.D.), after ergonovine .15 mg intravenously there was a significant fall in the peak to basal flow velocity response to the same dose of papaverine ( $4.1 \pm 1.9$  to  $3.4 \pm 1.6$ ,  $p < .05$ ) and a significant increase in the coronary resistance index ( $30.5 \pm 13.9$  to  $37.6 \pm 15.2$ ,  $p < .05$ ). Proximal left anterior descending artery dimensions after ergonovine did not change compared to after papaverine. Further, the 4 patients who had a decrease in peak to basal flow velocity after ergonovine were the same 4 who had chest pain and decreased great cardiac vein flow during pacing after ergonovine, compared to pacing alone, major criteria for microvascular angina.

Thus, ergonovine decreases peak pharmacologic flow reserve in patients with microvascular angina, indicating vasoconstriction of the coronary microcirculation.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 04803-01 CB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Elderly patients with hypertrophic cardiomyopathy

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

Jannet F. Lewis, M.D.

Special Volunteer/Guest Researcher

CB NHLBI

Barry J. Maron, M.D.

Senior Investigator

CB NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Cardiology Branch

## SECTION

Echocardiology Laboratory

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

0.6

## PROFESSIONAL:

0.6

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This report describes a subgroup of 52 elderly patients with obstructive hypertrophic cardiomyopathy in whom certain clinical and morphologic features differed importantly from many other patients with this disease. Ages ranged from 60-84 years (mean 69) and the vast majority (45 or 87%) were women.

Echocardiographic examination showed relatively small hearts having only modest ventricular septal hypertrophy, associated with marked distortion of left ventricular outflow tract morphology. Sizeable deposits of calcium in the mitral annulus appeared to contribute to the outflow tract narrowing. In most elderly study patients, anterior excursion of the mitral valve leaflets was relatively restricted, and the systolic apposition between mitral valve and septum resulted from a combination of anterior motion of the mitral valve and posterior excursion of the septum.

The vast majority of the patients (50 of 52) remained symptomatic (or only mildly symptomatic) for most of their lives and often did not develop severe and intractable symptoms until the sixth or seventh decade (ages 56-81 years; mean 66). Only 12 were improved by pharmacologic therapy; however, 14 of the 18 patients who underwent ventricular septal myotomy-myectomy or mitral valve replacement obtained symptomatic benefit from operation.

In conclusion, obstructive hypertrophic cardiomyopathy in many elderly (and predominantly female) patients may assume a distinctive morphologic appearance and a progressive clinical course.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 04804-01 CB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Hypoxia increases PDGF-like and basic FGF-like endothelial cell mitogenicity

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

Gordon Hafner, M.D.	Special Volunteer	CB	NHLBI
Michael Lee, B.S.	Special Volunteer	CB	NHLBI
Edith Speir, B.S.	Biochemist	CB	NHLBI
Ward Cascells, M.D.	Senior Investigator	CB	NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB	NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Cardiology Branch

## SECTION

Experimental Physiology and Pharmacology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

0.9

## OTHER:

0.1

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Basic fibroblast growth factor (bFGF) is a polypeptide mitogen for endothelial cells and a variety of other mesenchymal cells. It also speeds wound healing and produces angiogenesis in bioassays, but it is not known whether the results of adding FGF mean that FGF is involved in these functions naturally. It is not known what regulates FGF transcription, translation or release. Considerable evidence suggests that hypoxia can promote angiogenesis and many mechanisms have been proposed for this effect. One possibility would be that hypoxia promotes the release of basic FGF from hypoxic or ischemic tissue. Since endothelial cells synthesize FGF, at least in culture, they could be the cellular source of FGF. To examine this possibility we subjected confluent mouse lung capillary endothelial cells to 5% oxygen for 3 hours a day for 4 days. This stimulus did not cause cell death or an increase or decrease in replication. However, it increased the growth activity in the conditioned medium for 3T3 fibroblast. The increase was found in the fractions that eluted at 0.5 and at 2.0 of MNaCl, which is consistent with an increase in PDGF and basic FGF in the conditioned medium of the hypoxic cells. The cell lysates also showed increases in these fractions. Confirmation of the identity of the 2.0 M fractions was obtained with a competitive equilibrium radioimmunoassay, using an antiserum raised against the first 24 amino acids of basic FGF. These results are consistent with either an increase in the synthesis and release of these peptides or possibly a decrease in their proteolytic degradation, both within cells and in the medium, in response to hypoxia and its concomitant acidosis. These findings are of potential relevance to wound healing, tumor growth and the development of collaterals in response to myocardial ischemia or infarction.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 04805-01 CB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Timing and locus of vascular proliferation in acute myocardial infarction

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Fernando Bazoberry, M.D.	Guest Researcher	CB	NHLBI
Edith Speir, B.S.	Biochemist	CB	NHLBI
Victor Ferrans, M.D.	Chief, Ultrastructure Section	PB	NHLBI
Ward Casscells, M.D.	Senior Investigator	CB	NHLBI

## COOPERATING UNITS (if any)

Pathology Branch, NHLBI

## LAB/BRANCH

Cardiology Branch

## SECTION

Experimental Physiology and Pharmacology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

0.5

## PROFESSIONAL:

0.4

## OTHER:

0.1

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Despite increasing evidence that coronary collaterals can prevent or limit the size of myocardial infarction, it is not known how these collaterals develop. Whereas normal human pig and rat hearts have only a small number of coronary to coronary collaterals, 3 to 40 microns in size, normal dog heart collaterals are numerous and are 100-200 microns in size. In response to progressive narrowing of a coronary vessel over 5 days, canine vessels not only dilate but grow larger by a process involving cell division in all 3 layers of the vessel wall. It is not known whether ischemia triggers small vessel proliferation that is seen in embryogenesis and tumor angiogenesis. In humans with coronary artery disease, large interarterial collaterals are sometimes seen but it is not known whether these are due to expansion of pre-existing interarterial arterioles or whether they represent the final process of vascular remodeling following capillary proliferation. Therefore, as a preliminary step to studying the vasoproliferative response to ischemia without infarction, we studied the early proliferative response to acute myocardial infarction produced by coronary ligation in rats. In the nonoperated and sham operated controls we were able to confirm the very low level of cell proliferation in the normal heart. However, an increase in labeling began by 48 hours in the border zone of the infarct, in fibroblasts and capillary and venular endothelial cells. By 48 hours there was intense labeling which extended into the non-inflamed nonischemic tissue, where it was seen only in the capillaries and venules. Thus, these data suggest that in myocardial infarction neovascularization begins very early and is found mostly in capillaries and venules as is the case in tumor angiogenesis. This suggests that the cellular physiology and biochemistry of angiogenesis are applicable to myocardial infarction. Furthermore, these growth responses occur sufficiently quickly to influence such events as infarct extension, expansion or rupture. Current studies are designed to see if this is true of myocardial ischemia as well.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

201 HL 04806-01 CB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Delayed increase in smooth muscle cell calcium with basic FGF

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Yi Fu Zhou, M.D.	Guest Researcher	CB	NHLBI
Ward Casscells, M.D.	Senior Investigator	CB	NHLBI
Antonio Bartorelli, M.D.	Guest Researcher	CB	NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB	NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Cardiology Branch

## SECTION

Experimental Physiology and Pharmacology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

1

## PROFESSIONAL:

0.9

## OTHER:

0.1

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

When growth factors cause cells to proliferate, the earliest signal is usually an increase in cytosolic calcium and pH. The increase in calcium lasts for seconds to minutes. However, there are exceptions. Some cells which proliferate in response to EGF or to insulin are characterized only by phosphorylation of tyrosines and not by a calcium signal. Smooth muscle cell contraction is also usually initiated by an increase in cytosolic calcium, which may be transient or sustained. An exception is the slow increase in contraction in response to phorbol esters. Several groups, including our own, have noted that vasoconstricting agents tend to act as weak mitogens, whereas some vasodilating drugs, including calcium antagonists, inhibit growth at least at high doses. Conversely, there is a recent report of vasoconstrictive activity of EGF, PDGF, and basic FGF. These intriguing connections between cell contraction and cell proliferation may have some relevance to the clinical situations where growth and contraction (or tension) are associated such as hypertension and hypertrophy.

Because we have found basic FGF in cultured endothelial and smooth muscle cells and because an increase in cytosolic calcium has been noted with FGF administration in several cell types, such as endothelial cells and some fibroblasts, we anticipated that it would cause an early calcium signal in cultured smooth muscle cells. However, we found no such signal. We did see an increase to 170 nM (from 100 nM baseline) some time between 12 and 24 hours after bFGF was added. This is an average value in a suspension of cells, and so could indicate each cell developed a modest increase, or that the mean was weighted by an increase in the number of cells in M phase, which is characterized by calcium transients. Studies are in progress to distinguish between these possibilities. In addition, these studies are being repeated with freshly enzyme-dispersed cells which, in contrast to passaged cells or explant-cultured cells, retain their ability to contract.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 04807-01 CB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Verapamil influences growth of cultured vascular smooth muscle cells

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

C. Phillip Nesbitt, M.D.	Special Volunteer	CB	NHLBI
Yi Fu Zhou, M.D.	Guest Researcher	CB	NHLBI
Ward Casscells, M.D.	Senior Investigator	CB	NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Cardiology Branch

## SECTION

Experimental Physiology and Pharmacology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

0.6

## PROFESSIONAL:

0.5

## OTHER:

0.1

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

Calcium antagonist drugs, such as verapamil, have recently been shown to have some inhibitory effects on the development of atherosclerosis and of the smooth muscle hyperplasia in hypertension. Some of this has been attributed to decreases in blood pressure and to antiplatelet effects. Verapamil has also been shown to alter metabolism of low density lipoproteins. However, because calcium is a requirement for cell growth, which can be inhibited by lowering the extracellular calcium concentrations in vitro, studies have recently looked for direct inhibition of cell growth by calcium antagonists. Verapamil and nifedipine have been found to inhibit the growth of cultured vascular smooth muscles at  $10^{-5}$  to  $10^{-6}$  M but effects at physiologic range ( $10^{-7}$ ) were slight. Those studies were performed in the presence of 10% serum, using rapidly growing cells and cells made quiescent by starvation. We took another approach and studied cells made quiescent by starvation, but refed with zero to 0.1% serum. We found a 6-fold increase in thymidine incorporation in these cells when given verapamil at  $10^{-8}$  to  $10^{-7}$  M. Inhibition was seen at  $10^{-5}$  M verapamil when cells were refed with 10% serum no effect was observed until  $10^{-5}$  M, at which point inhibition was seen as in the previous studies. This novel observation suggests two hypotheses: 1) that starved quiescent smooth muscle cells are deficient in ATP, cannot maintain the electrochemical calcium gradient and, because of calcium leakage into the cell, experience a form of calcium mediated growth inhibition which is preventable by verapamil; 2) there is a growth inhibitor signal in confluent starved cells which is mediated by calcium entry and this entry is through voltage dependent channels and hence prevented by verapamil.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 HL 04808-01 CB
PERIOD COVERED October 1, 1987 to September 30, 1988		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Transforming growth factor beta-1 in normal heart and in infarction		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
Fernando Bazoberry, M.D.	Special Volunteer	CB NHLBI
Nancy Thompson, M.D.	Medical Staff Fellow	LCB NCI
Michael Sporn, M.D.	Chief, Laboratory of Chemoprevention	LCB NCI
Edith Speir, B.S.	Biochemist	CB NHLBI
Ward Casscells, M.D.	Senior Investigator	CB NHLBI
Victor Ferrans, M.D.	Chief, Ultrastructure Section	PB NHLBI
Kathy Flanders, M.D.	Medical Staff Fellow	LCB NCI
COOPERATING UNITS (if any) Laboratory of Chemoprevention, NCI Pathology Branch, NHLBI		
LAB/BRANCH Cardiology Branch		
SECTION Experimental Physiology and Pharmacology		
INSTITUTE AND LOCATION NHLBI, NIH, Bethesda, MD		
TOTAL MAN-YEARS: 1.0	PROFESSIONAL: 0.9	OTHER: 0.1
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Transforming growth factor beta<sub>1</sub> is a polypeptide originally isolated from platelets which is a regulator of cell growth and differentiation. In vitro, given certain cultured conditions TGF beta<sub>1</sub> can cause fibroblasts to grow in soft Agar - a property of transformed cells. More often, though, TGF beta is an inhibitor of growth, for example it inhibits FGF stimulation of endothelial cell proliferation. However, it cooperates with FGF in induction of the embryonic mesoderm. TGF beta<sub>1</sub> also regulates production of extracellular matrix, and in particular regulates plasminogen activators and the PA inhibitor, PAI-1. Plasmin can activate latent TGF beta<sub>1</sub>. All these features suggest that TGF beta<sub>1</sub> could play one or more important roles if present in the heart.</p> <p>In this study, we used a polyclonal antiserum raised by Flanders and Sporn against the first 30 residues of TGF beta 1. Specificity of the immunocytochemistry was indicated by the absence of staining with normal (non-immune) serum or with immune serum pre-absorbed with excise TGF beta 1. Immunohistochemical evidence of TGF beta<sub>1</sub> was found in cardiac myocytes and in smooth muscle cells and endothelial cells of sections of normal rat hearts. Supportive evidence was the presence of a 2.4 Kb mRNA transcript on northern blotting.</p> <p>Coronary ligations were then performed in these rats. Between 1 and 6 hours after ligation ir TGF beta-1 began to be lost from cardiac myocytes, sparing only 1-2 layers of cells in the subendocardium and around vessels, which are presumably viable cells. Interestingly, by 6 hours these cells seemed to actually have an increase in immunoreactivity, as did myocytes in the border zone. These effects suggest that TGF beta-1 may well be involved in fibrinolysis, wound healing or angiogenesis in the setting of myocardial infarction.</p>		



## NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 04809-01 CB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cardiac norepinephrine kinetics in hypertrophic cardiomyopathy

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

John E. Brush, M.D.	Senior Investigator	CB	NHLBI
Richard O. Cannon, M.D.	Senior Investigator	CB	NHLBI
Barry J. Maron, M.D.	Senior Investigator	CB	NHLBI
Julio A. Panza, M.D.	Guest Researcher	CB	NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB	NHLBI
Graeme Eisenhofer, Ph.D.	Visiting Fellow	HB	NHLBI
Moshe Garty, M.D.	Visiting Associate	HB	NHLBI
David S. Goldstein, M.D.	Senior Investigator	HB	NHLBI

## COOPERATING UNITS (if any)

Hypertension Branch, NHLBI

## LAB/BRANCH

Cardiology Branch

## SECTION

Cardiovascular Diagnosis Section

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

1

## PROFESSIONAL:

1

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We examined cardiac norepinephrine kinetics in 11 patients with hypertrophic cardiomyopathy (HCM) and in 10 controls. Neuronal uptake of norepinephrine was assessed by comparing 1) the extraction of labelled norepinephrine and isoproterenol, which is not a substrate for neuronal uptake; and 2) the relation between dihydroxyphenylglycol (DHFG) production, an intraneuronal metabolite of norepinephrine, and spillover of norepinephrine. Cardiac norepinephrine extraction was less in HCM patients than in C ( $59 \pm 17$  vs  $79 \pm 13\%$ ,  $p < 0.05$ ), whereas extraction of isoproterenol was similar ( $13 \pm 23$  vs  $13 \pm 14\%$ ). The arteriovenous difference in norepinephrine was larger in the HCM patients ( $73 \pm 77$  vs  $13 \pm 50$  pg/ml,  $p < 0.05$ ) as was the arteriovenous difference times blood flow ( $7.3 \pm 7.3$  vs  $0.8 \pm 3.0$  ng/min,  $p < 0.05$ ). The slope of the line relating cardiac DHFG production to cardiac norepinephrine spillover was less in the HCM patients than in controls ( $p < 0.005$ ), indicating that the increased norepinephrine arteriovenous difference in HCM is not due to increased norepinephrine release (which would result in increased DHFG production), but due to decreased norepinephrine neuronal uptake. These results indicate that cardiac norepinephrine neuronal uptake is defective in HCM. This defect may increase norepinephrine levels at myocardial adrenoceptors and thereby play a role in the pathophysiology of HCM.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 HL 04809-01 CB
PERIOD COVERED October 1, 1987 to September 30, 1988		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Cardiac norepinephrine kinetics in hypertrophic cardiomyopathy		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
John E. Brush, M.D.	Senior Investigator	CB NHLBI
Richard O. Cannon, M.D.	Senior Investigator	CB NHLBI
Barry J. Maron, M.D.	Senior Investigator	CB NHLBI
Julio A. Panza, M.D.	Guest Researcher	CB NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB NHLBI
Graeme Eisenhofer, Ph.D.	Visiting Fellow	HB NHLBI
Moshe Garty, M.D.	Visiting Associate	HB NHLBI
David S. Goldstein, M.D.	Senior Investigator	HB NHLBI
COOPERATING UNITS (if any)  Hypertension Branch, NHLBI		
LAB/BRANCH Cardiology Branch		
SECTION Cardiovascular Diagnosis Section		
INSTITUTE AND LOCATION NHLBI, NIH, Bethesda, MD		
TOTAL MAN-YEARS: 1	PROFESSIONAL: 1	OTHER:
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>             We examined cardiac norepinephrine kinetics in 11 patients with hypertrophic cardiomyopathy (HCM) and in 10 controls. Neuronal uptake of norepinephrine was assessed by comparing 1) the extraction of labelled norepinephrine and isoproterenol, which is not a substrate for neuronal uptake; and 2) the relation between dihydroxyphenylglycol (DHFG) production, an intraneuronal metabolite of norepinephrine, and spillover of norepinephrine. Cardiac norepinephrine extraction was less in HCM patients than in C (<math>59 \pm 17</math> vs <math>79 \pm 13\%</math>, <math>p &lt; 0.05</math>), whereas extraction of isoproterenol was similar (<math>13 \pm 23</math> vs <math>13 \pm 14\%</math>). The arteriovenous difference in norepinephrine was larger in the HCM patients (<math>73 \pm 77</math> vs <math>13 \pm 50</math> pg/ml, <math>p &lt; 0.05</math>) as was the arteriovenous difference times blood flow (<math>7.3 \pm 7.3</math> vs <math>0.8 \pm 3.0</math> ng/min, <math>p &lt; 0.05</math>). The slope of the line relating cardiac DHFG production to cardiac norepinephrine spillover was less in the HCM patients than in controls (<math>p &lt; 0.005</math>), indicating that the increased norepinephrine arteriovenous difference in HCM is not due to increased norepinephrine release (which would result in increased DHFG production), but due to decreased norepinephrine neuronal uptake. These results indicate that cardiac norepinephrine neuronal uptake is defective in HCM. This defect may increase norepinephrine levels at myocardial adrenoceptors and thereby play a role in the pathophysiology of HCM.           </p>		





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 04810-01 CB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cardiac myocytes express acidic and basic FGF

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Michael S. Lee, B.S.	Special Volunteer	CB	NHLBI
Yi Fu Zhou, M.D.	Guest Researcher	CB	NHLBI
Edith Speir, B.S.	Biochemist	CB	NHLBI
Ward Casscells, M.D.	Senior Investigator	CB	NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Cardiology Branch

## SECTION

Experimental Physiology and Pharmacology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

0.5

## PROFESSIONAL:

0.4

## OTHER:

0.1

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Acidic and basic fibroblast growth factors (aFGF and bFGF) are mitogenic peptides which also have angiogenic, neurotropic, and morphogenic activities. They have been found in most tissues but their cellular origin in vivo is unknown. Approximately half of all mesenchymal cells synthesize bFGF in culture and a minority of these cells synthesize aFGF in culture. However, the in vitro expression may not be a true indicator of the situation in vivo. The absence of immunocytochemistry or in situ hybridization for these peptides and genes, respectively, has prevented their cellular localization.

We took another approach and isolated cardiac myocytes by enzymatic digestion. These cells were then washed, lysed and subjected to heparin sepharose chromatography. The fractions which elute in the positions characteristic of aFGF and bFGF were then tested for mitogenic activity on 3T3 cells and tested for immunoreactivity for acidic and basic FGF by dot blot. Both types of assays suggest that these peptides are abundant in cardiac myocytes. The roles of these peptides in young adult rat cardiac myocytes, which are not dividing and not subject to pathologic hypertrophy or to angiogenesis, will require further investigation.

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Annual Report of the Laboratory of Cell Biology  
National, Heart, Lung, and Blood Institute  
October 1, 1987 to September 30, 1988

This summary describes some of the highlights contained in the 9 individual project reports of the 7 independent investigators of the Laboratory of Cell Biology.

Non-muscle Myosins: Intensive study of Acanthamoeba myosin I, the prototype of a new class of myosin isoenzymes, continues. Previously, Dr. Korn had described two myosin I isoenzymes (A and B) each with a single heavy chain and a single light chain (140 and 127 kDa, and 17 and 27 kDa, respectively). Now a third Acanthamoeba myosin I isoenzyme has been characterized with a heavy chain of 130 kDa and 1 or 2 light chains of about 14.5 kDa. Dr. Hammer has cloned and sequenced a cDNA which also is myosin I-like and differs from IA and IB; in the absence of protein sequence, it is not yet known if this cDNA corresponds to the third myosin I isoenzyme that has been purified or represents a ; fourth myosin I isoenzyme.

Myosin I is similar to conventional myosins (myosin II) in its enzymatic properties but differs in its inability to form filaments and its ability to cross-link actin filaments. These two differences derive from the substitution of a novel, relatively short C-terminal sequence (with a second actin-binding site) for the long helical C-terminal portion of the heavy chain that is responsible for filament formation by conventional myosins. The similarities in catalytic activities of myosin I and II, including actin-activated ATPase activity, derive from the similar sequences of the N-terminal 80-90 kDa. Dr. Korn has obtained considerable information about the functional organization of the myosin I heavy chain by a combination of specific cross-linking procedures and gel overlay techniques with specific proteolytic and chemical cleavages. In summary, the adenine moiety of ATP binds about 12 kDa from the N-terminus, the regulatory phosphorylatable amino acid (threonine for IA and serine for IB) is at about 38 kDa, the actin-binding site involved in catalysis includes three regions at 58-64, 64-73, and 73-80 kDa, and the second actin-binding site is between 112-127 kDa. These assignments are consistent with, and extend, the assignments for skeletal muscle myosin.

The physiological role of myosin I is an important, and as yet unanswered, question. Double immunofluorescence studies have shown that myosin I co-localizes with actin in a punctate distribution at the cell surface but does not extend, as actin does, down microspikes. Myosin I is associated with purified plasma membranes as a non-integral membrane protein, but is not linked through actin. Membranes stripped of myosin I, and most of their actin, re-bind myosin I in a saturable manner with a  $K_D$  of about  $0.05 \mu M$ . Immunofluorescence studies with anti-Dictyostelium myosin I indicate that myosin I is located in the leading edge of migrating cells. This region is rich in actin filaments but devoid of conventional myosin II which suggests the possibility of a specific role for myosin I in ameboid movement.



Acanthamoeba myosin II is a conventional myosin whose actin-activated ATPase activity is inhibited by phosphorylation of 3 serine residues that occur in a 29-amino acid non-helical tailpiece of the 2 heavy chains. Previous work has led to the hypothesis that this regulation involves a change in conformation of the myosin II bipolar filaments, perhaps involving a "hinge" region (identified by sequence and electron microscopy) that occurs about 40% in from the C-terminus of the heavy chain. Removal of a C-terminal 66-residue peptide from the heavy chains abolishes filament formation and actin-activated ATPase activity. One approach to this problem, being pursued by Dr. Korn, is to determine whether the regulatory (unphosphorylated) serines are required for activity. Thus far it has been possible to remove a portion of the non-helical tailpiece including 2 of the 3 serines by selective proteolysis. This modified myosin II has essentially normal actin-activated ATPase activity that is inactivated by phosphorylation of the remaining regulatory serine.

An alternative approach to this problem is being pursued by Dr. Hammer. The entire myosin II rod has been successfully expressed in E. coli and the isolated rod shown to be capable of re-folding and associating into filaments. This will allow future studies in which amino acid replacements will show the essential features that are required for a functioning hinge, and, by co-polymerization with native myosin II, how alterations in the hinge region affect actin-activated ATPase activity.

At the protein level, only one isoform of Acanthamoeba myosin II has been identified, but Dr. Hammer has cloned and now completed the sequence of a genomic DNA that appears to represent another myosin II. S1 mapping experiments indicate that this is a functional, transcribed gene but its cDNA (or the corresponding protein) has yet to be identified.

Actin: Many of the details of the elongation phase of actin polymerization are reasonably well established, but the initial steps in which monomer is converted to a nucleus and then to short oligomers are still essentially unknown. Recent work by Dr. Korn has established that actobindin (an actin-binding protein from Acanthamoeba that is a very potent inhibitor of the early phases of actin polymerization) binds reversibly to and inhibits the elongation of an intermediate between monomeric G-actin and polymeric F-actin. This would require that there be an intermediate oligomeric actin species with properties different than those of actin monomer or actin polymer.

Microtubule Cytoskeleton: Microtubule-microtubule and microtubule-membrane associations are less well defined than those involving microfilaments. Dr. Flavin has been studying the peripheral corset of microtubules that underlie the plasma membrane of Crithidia fasciculata. Three potential microtubule cross-linking proteins has previously been identified: COP-33, COP-40 and COP-61, each named according to its subunit Mr. Native COP-61 has now been found to be a dimer and the other two appear to be tetramers in their native forms.



Each binds to microtubules in vitro with a  $K_D$  of about 0.1-0.4  $\mu M$ , with some positive cooperativity.

Immunoelectron microscopy using specific polyclonal antibodies and gold-labeled secondary antibodies indicate that COP-61 is associated with microtubules in situ. COP-33 has not yet been detected by this procedure. COP-40 appears to be localized in the glycosome, an organelle unique to trypanosomatids, which contains glycolytic enzymes and, therefore, may not function as a microtubule-binding protein in situ.

Heat-shock Proteins and Uncoating ATPase: A number of proteins, most prominently a group of Mr 70,000, appear or greatly increase in amount when cells are stressed by an increase in temperature. The 70-kDa proteins are thought to bind to other proteins either to disaggregate them or maintain them in a disaggregated state. The best studied example is the 70-kDa uncoating ATPase which, in the presence of ATP, removes the protein clathrin from the cytoplasmic surface of coated vesicles. Pioneering work by Dr. Rothman with artificial clathrin vesicles indicated that the uncoating ATPase acted catalytically solubilizing multiple clathrin molecules for each molecule of uncoating ATPase.

Drs. Eisenberg and Greene have studied the action of the uncoating ATPase on intact coated vesicles isolated from bovine brain. Their data indicate that the uncoating ATPase acts stoichiometrically with three 70-kDa proteins binding to the clathrin trimer (clathrin comprises three 180-kDa and three 30-kDa polypeptides) resulting in a soluble ATPase-clathrin complex. Once all of the uncoating ATPase molecules are bound to clathrin no further dissociation of clathrin occurs and the ATPase activity of the enzyme is inactivated.

Soluble clathrin does not, however, bind to or inactivate the ATPase activity of the uncoating ATPase. This indicates that during the uncoating process either the ATPase or the clathrin is altered so as to increase the affinity between the two proteins. The ATPase-clathrin complex that forms when the uncoating ATPase is incubated with coated vesicles can be dissociated by lowering the pH from 7 to 6.5 (and by other methods) and the enzyme can then uncoat additional coated vesicles. This demonstrates that irreversible inactivation of the uncoating ATPase does not occur.

In a recent observation, it was found that incubating the coated vesicles with phosphatase inhibits their subsequent uncoating by the ATPase. This, and other, evidence suggests that one or more proteins of the coated vesicles must be phosphorylated for the uncoating ATPase to act.

Bioenergetics: Last year, Dr. Hendler described a new redox potential for cytochrome  $a_3$  at about 780 mV. Now, he has established that there are also two low-potential forms of cytochrome  $a_3$ , one with an absorption peak at 429 nm and one with a peak at 448 nm; both forms





have a mid-point potential of about 180 mV. The 429-form appears to be more stable but to convert the 448-form to the 429-form requires that the enzyme be incorporated into liposomes. This suggests that the 429-form is the functional form in situ.

Dr. Hendler is also continuing his work on developing microcomputer-controlled systems to measure electron and proton fluxes and the membrane potential and pH gradient across energy-transducing membranes that arise during respiration. A system of major interest, mitochondria, present a special problem because the mitochondrial volume changes with the state of energization. It has been found possible to correct for this by simultaneous measurements of light scattering and the use of a standard curve relating light scattering to internal volume measured by appropriate radioactive molecules.

Pure cytochrome oxidase has been incorporated into phospholipid : liposomes. The enzyme shows a very high degree of respiratory control (i.e.  $O_2$  consumption ceases when the cytochrome is energized) equivalent to the best preparations of mitochondria. The vesicles pump protons from inside to outside coupled to the transport of electrons from cytochrome c to  $O_2$  and they establish a pH gradient and membrane potential. This reconstructed system should allow the elucidation of mechanistic details of these integrated reactions.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00401-22 LCB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Thermodynamic studies of electron and proton affinities of cytochromes

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Richard W. Hendler

Section Head

LCB, NHLBI

Others: Gurmel S. Sidhu

Visiting Fellow

LCB, NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Cell Biology

## SECTION

Membrane Enzymology

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.5

## PROFESSIONAL:

1.5

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

It has been established that mammalian cytochrome a3 has two low potential forms in addition to a high potential form described in last year's report. Both low potential forms have an  $\alpha$  peak at 602 nm, but are distinguished by their Soret peak; one at 429 nm and one at 448 nm. The a3(429) is thermodynamically more stable than the a3(448), but it is formed very slowly unless three requirements are met. There must be extra lipids and proteins present (i.e. egg homogenate), K3Fe(CN)6 at ~0.5 mM, and an exposure to -400 mV for 1 hr. If the enzyme is incorporated into a proteoliposome membrane, the requirement for egg homogenate is removed. Therefore, the a3(429) form must represent the biologically significant species. Both forms are capable of forming CO complexes (CO is analogous to O2). The Em's of both a3(448) and a3(429) are near 180 mV as established by both oxidative and reductive titrations. The Em of a3(448)·CO is near 350 mV and the Em of a3(429)·CO is near 250 mV. Both species can be oxidized using K3Fe(CN)6 as the oxidizing agent. This finding was not anticipated on the basis of past work. However, in these earlier studies, K3Fe(CN)6 was added at a very low temperature because of the concern that O2 might displace CO from the enzyme at room temperature. Our experiments were performed anaerobically at room temperature.







DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 HL 00419-08 LCB
PERIOD COVERED October 1, 1987 to September 30, 1988		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Structure-function relationships in Eukaryotic cells		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI: Blair Bowers	Research Biologist	LCB, NHLBI
Others: Thomas Olszewski	Biologist	LCB, NHLBI
COOPERATING UNITS (if any)  None		
LAB/BRANCH Laboratory of Cell Biology		
SECTION Cellular Biochemistry and Ultrastructure		
INSTITUTE AND LOCATION National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS: 2	PROFESSIONAL: 2	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>             We are studying the exchange of membrane between the surface and the internal vacuolar system that occurs during endocytosis. As one aspect of this study, we are examining the distribution of membrane peptides with the aid of monoclonal antibodies that we have raised to <u>Acanthamoeba</u> membranes. One monoclonal, MC1, is specific to a carbohydrate epitope that occurs on a number of membrane peptides. A second monoclonal, MC3, reacts with peptide(s) that run as a small cluster of bands on SDS gels. Freeze-fracture replicas of cells labeled simultaneously with both antibodies and a different size colloidal gold probe for each antibody, showed that the antigens are uniformly distributed and intermingled in the plane of the membrane. Fluorescent labeling showed that both epitopes are present in all internal vacuole membranes as well. We worked out procedures for immunolabeling cells embedded in plastic in order to quantitatively assess antigen distribution in internal membranes relative to the plasma membrane. Preliminary measurements of label distribution in electron micrographs indicate that MC1 antigens are more concentrated in the vacuolar membrane and MC3 antigens are more concentrated in the plasma membrane. We are investigating whether differential uptake or return of antigens during the membrane recycling that accompanies endocytosis can account for this distribution.           </p>		

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00501-15 LCB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Actin Polymerization

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Edward D. Korn Chief LCB, NHLBI

Others: Peter K. Lambooy Staff Fellow LCB, NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Cell Biology

## SECTION

Cellular Biochemistry and Ultrastructure

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.2

## PROFESSIONAL:

1.2

## OTHER:

0

## CHECK APPROPRIATE BCX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Actobindin, a 25,000-dalton dimeric protein purified from Acanthamoeba castellanii was previously shown to form a 1:1 molar complex with both Acanthamoeba and rabbit muscle G-actin with KD values of about 5  $\mu$ M and 7  $\mu$ M, respectively, and not to interact with F-actin (Lambooy, P. K., and Korn, E. D. (1986) J. Biol. Chem. 261, 17150-17155). We now find that actobindin is a much more potent inhibitor of the early phases of polymerization of both Acanthamoeba and muscle G-actin than can be accounted for by its binding to G-actin. Actobindin inhibits the polymerization of both G-ATP-actin and G-ADP-actin, and has little, if any, effect on the rate of ATP hydrolysis that accompanies polymerization of G-ATP-actin. The kinetics of actin polymerization in the presence of actobindin are qualitatively consistent with the postulation that actobindin binds reversibly to and inhibits the elongation of an intermediate between G-actin and F-actin, perhaps a small oligomer(s), or a species in equilibrium with such an intermediate. This hypothesis implies the, at least transient, existence of an actin species with properties different than those of monomers and filaments. Actobindin may, then, provide a useful experimental tool for investigating the still relatively obscure early steps in actin polymerization. Irrespective of its mechanism of action, actobindin might serve in situ to reduce the rate of actin polymerization de novo while having relatively little effect on the rates of elongation of existing filaments or from actobindin-resistant nucleating sites.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 HL 00503-16 LCB
PERIOD COVERED October 1, 1987 to September 30, 1988		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Structure, assembly and function of microtubules		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI: Martin Flavin	Section Head	LCB, NHLBI
Others: Sulie Chang	Staff Fellow	LCB, NHLBI
Ravi Kambadur	Visiting Fellow	LCB, NHLBI
Gregory Bramblett	Research Assistant	LCB, NHLBI
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Cell Biology		
SECTION Organelle Biochemistry		
INSTITUTE AND LOCATION National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS: 3	PROFESSIONAL: 3	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>             The Trypanosomatid cytoskeleton consists of a corset of regularly spaced microtubules linked to each other and to the closely overlying plasma membrane. We are interested in the composition of the cross-links and in how microtubules are bound to membranes. We have initially focussed on 3 proteins (designated, relative to their subunits Mr, COP-33, COP-40 and COP-61) because of their prominence in the isolated cytoskeleton, and because 2 of them also cross-linked microtubules to each other <u>in vitro</u>. We have purified each and noted the following properties, listed in the above protein sequence. Detergent needed for solution: yes, no, yes. Oligomeric state in solution: tetramer, tetramer, dimer. Crosslink microtubules <u>in vitro</u> (periodicity/length in nm): 8/12, 8/18, none. Binding to microtubules: maximum mol/mol - 0.3, 0.6, 0.3; apparent Kd (<math>\times 10^{-7}</math> M) - 3.2, 3.7, 1.3; positive cooperativity - yes, yes, yes.           </p> <p>             Rabbit antisera to each COP, adequate in titer and specificity, have been used together with gold-labeled secondary antibodies to begin to localize the antigens within these very small cells. COP-41 antibody bound to glycosomes, using thin sections from etched soft resin, and we believe it is a glycolytic enzyme which binds to the corset apparatus only after cell disruption. With this type of cell preparation the COP-33 antigen has not yet been detected. Gold-labeling by anti COP-61 appears similar to that by antitubulin, infrequent but concentrated in the corset-plasma membrane region.           </p>		



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 HL 00506-13 LCB
PERIOD COVERED October 1, 1987 to September 30, 1988		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Acanthamoeba myosins and kinesin		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI: Edward D. Korn                      Chief                      LCB, NHLBI  Others: Hanna Brzeska, Visiting Fellow, LCB, NHLBI; Thomas Lynch, Staff Fellow, LCB, NHLBI; Ray Scharff, Chemist, LCB, NHLBI; Hidetake Miyata, Visiting Fellow, LCB, NHLBI; Chhanda Ganguly, Staff Fellow, LCB, NHLBI; Venugopal Sathyamoorthy, Staff Fellow, LCB, NHLBI		
COOPERATING UNITS (if any)  None		
LAB/BRANCH Laboratory of Cell Biology		
SECTION Cellular Biochemistry and Ultrastructure		
INSTITUTE AND LOCATION National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS: 6.5	PROFESSIONAL: 6.5	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>Structure-function studies have shown that at least 2 of the 3 regulatory, phosphorylatable serines can be proteolytically removed from the non-helical tailpiece of the <u>Acanthamoeba</u> myosin II heavy chain with essentially no effect on filament formation or phosphorylation-regulatable actin-activated ATPase activity.</p> <p>The positions of the ATP binding site, phosphorylation site and two different actin-binding sites have been localized in <u>Acanthamoeba</u> myosin IA and IB. The phosphorylated amino acid in IA was shown to be threonine while it is serine in IB. The sequence around the PThr has been determined. A third <u>Acanthamoeba</u> myosin I isoform has been purified and characterized. Myosin I has been shown to be reversibly bound (<math>K_d \sim 0.05 \mu M</math>) to the plasma membrane of <u>Acanthamoeba</u> through saturable sites that appear to be independent of actin and from which the myosin can be dissociated at high ionic strength. Studies with <u>Dictyostelium</u> suggest an important role for myosin I at the leading edge of motile cells, a region that is devoid of myosin II but is enriched with actin filaments.</p>		

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00514-05 LCB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The structure and function of nonmuscle myosins

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: John A. Hammer III	Research Biologist	LCB, NHLBI
Others: Goeh Jung	Visiting Associate	LCB, NHLBI
David Halsall	Visiting Fellow	LCB, NHLBI
Jill Horowitz	Guest Researcher	MDA
Edward D. Korn	Chief, LCB	LCB, NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Cell Biology

## SECTION

Cellular Biochemistry and Ultrastructure

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

3.9

## PROFESSIONAL:

3.9

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The purpose of this project is to isolate genes encoding the heavy chains of nonmuscle myosins and to use these genes as tools to investigate myosin structure/function relationships and the in vivo functions of nonmuscle myosins. Using molecular cloning techniques, we previously isolated from the soil amoeba Acanthamoeba castellanii the heavy chain genes for myosin II (a nonmuscle myosin possessing conventional structure) and myosin IB (an unusual, low-molecular weight, non-filamentous nonmuscle myosin). In this report we describe the cloning and characterization of two additional amoeba myosin heavy chain genes and our initial efforts at cloning a gene encoding a vertebrate form of myosin I. The significance of this work is that (i) the deduced protein sequences are of great value in furthering our understanding of the structural and functional properties of these myosins and (ii) by using the tools of molecular biology we can approach the study of these proteins in novel ways which are not possible using the classical techniques of protein chemistry. For example, we can dissect at the molecular level the mechanisms of enzymatic regulation using site-directed mutagenesis to alter the myosin molecules and we can examine the physiological roles of these myosins by reintroducing the genes back into cells. Both of these approaches are currently underway.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00515-03 LCB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Personal Workstation Project for Scientists

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Richard W. Hendler  
Richard I. ShragerSection Head  
MathematicianLCB, NHLBI  
LAS, DCRTOthers: David Songco  
Brian CollettChief, Pers. Work Station Office, DCRT  
Biophysicist  
LPB, NIADDK

## COOPERATING UNITS (if any)

Alan M. Demerle, Chief, Computer Systems Lab, DCRT; Perry Plexico, Chief, Project Development Section, CSL, DCRT; Keith L. Gorlen, James S. Del Priore and James Sullivan, CSL, DCRT

## LAB/BRANCH

Laboratory of Cell Biology

## SECTION

Membrane Enzymology

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

0.1

## PROFESSIONAL:

0.1

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A 16-bit microcomputer (PC/AT) can control complex experiments, collect large quantities of data, display the raw information, perform computations and display transformed data in real time. A mathematical modeling language "DAL" developed by Brian Collett can perform much of the same kind of analyses provided by MLAB. A test case employed a data matrix with ~15000 numbers in double precision (~120000 bytes). These data were subjected to SVD and the simultaneous fitting, with weights, of 47 parameters to 6 columns of data. The time to accomplish this was as fast as that used on the DEC 10 system and the results were the same. However, in order to use "DAL" a user must be proficient in partial differential equations and computer programming. For this reason, it is recommended that scientists wait 1 to 2 years until a revised form of MLAB will be available for use on PC's. An independent effort by LAS of DCRT has been centered on the use of the 32 bit personal work station provided by a Sun microcomputer. The mathematical modeling has been performed with "PC MATLAB". Similar problems for the scientist are presented with this package, as were cited above for "DAL".



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00516-02 LCB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

70-kDa Heat Shock Proteins and the Homologous Uncoating ATPase

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Evan Eisenberg	Section Head	LCB, NHLBI
Lois E. Greene	Research Chemist	LCB, NHLBI

Others: José Biosca, Visiting Associate; Samuel Chacko, IPA; John A. Evans, Staff Fellow; Bao-chong Gao, Visiting Fellow; Kondury Prasad, Visiting Associate; Sudhir Srivastava, Staff Fellow, LCB, NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Cell Biology

## SECTION

Cellular Physiology

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

6.8

## PROFESSIONAL:

6.8

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The overall focus of our laboratory is the study of the 70-kDa heat shock proteins and their role in both normal cellular processes and heat shock. First, we are investigating one of the only defined functions of a 70-kDa heat shock protein--the ability of the 70-kDa uncoating (UC) ATPase isolated from bovine brain to remove clathrin from clathrin coated vesicles in an ATP dependent reaction. In contrast to earlier reports suggesting that the UC ATPase catalytically removes clathrin from coated vesicles, our current results suggest that the UC ATPase removes clathrin stoichiometrically with one enzyme molecule binding to each of the three clathrin legs. The resulting enzyme-clathrin complex is stable for at least 24 hours in solution, and the bound enzyme is not able to uncoat freshly added coated vesicles. In addition to binding clathrin tightly, we also have evidence that the UC ATPase binds ADP extremely tightly which may explain why, in contrast to the many other ATPases, the 70-kDa proteins bind so tightly to ATP affinity columns. Surprisingly, in contrast to the tight binding of the enzyme to clathrin which it has dissociated from coated vesicles, the enzyme does not appear to bind to free clathrin in solution, suggesting that a special kind of complex is forming when the UC ATPase dissociates clathrin from coated vesicles. We also have evidence that the uncoating reaction may be controlled by phosphorylation of the coated vesicles. In addition to these studies on bovine brain UC ATPase, we have investigated the ability of the 70-kDa proteins isolated from yeast to uncoat bovine brain clathrin coated vesicles. Our results show that, the yeast 70-kDa proteins are much less effective than the bovine brain UC ATPase; 5 to 10-fold more yeast enzyme is required to carry out the same amount of uncoating as carried out by the brain enzyme. Since the yeast 70-kDa proteins are composed of several isoenzymes, we are investigating whether this low uncoating activity is due to full activity of only one of these isoenzymes, or whether the yeast proteins, in general, are much less active than the brain UC ATPase.

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ANNUAL REPORT OF THE  
LABORATORY OF CELLULAR METABOLISM  
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

October 1, 1987 to September 30, 1988

Research in the Laboratory of Cellular Metabolism is largely focused on the guanine nucleotide-binding (G) proteins that function in the adenylyl cyclase and other systems to transmit signals from the exterior of the cell to internal effectors. The objective of this effort is to elucidate mechanisms for control of synthesis, assembly and operation of these ubiquitous regulatory proteins. Major subjects of current studies are  $G_o$ , a G protein whose physiological role is at present unclear, and several recently recognized, so-called ADP-ribosylation factors that appear to be members of a different family of guanine nucleotide-binding proteins. In addition, work is continuing on characterization of specific cyclic nucleotide phosphodiesterase that play an important regulatory role in cells.

1.  $G_{o\alpha}$ : a Guanine Nucleotide-Binding Protein Involved in Signal Transduction.

$G_o$  is one of a family of guanine nucleotide-binding (G) proteins that function in several types of transmembrane signalling systems to couple cell surface receptors with an intracellular enzymatic response. Like the other G proteins,  $G_o$  is a heterotrimer of  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits. The  $\alpha$  subunits, which are different in each G protein, bind and hydrolyze GTP. They interact with both receptor and effector. Last year, we reported characterization of a cDNA clone for the  $\alpha$  subunit of  $G_o$  ( $G_{o\alpha}$ ) and its expression in E. coli. Oligonucleotide-directed single point mutations in the  $G_{o\alpha}$  cDNA are being used for structure-function studies. Two mutant proteins have now been characterized. In one, cysteine 351, which is the site of pertussis toxin-catalyzed ADP-ribosylation, is replaced by glycine. In the other, aspartic acid is substituted for glycine 352, immediately adjacent to the ADP-ribosylation site. Mutations were confirmed by sequencing segments of the DNA. The mutant proteins, isolated from the E. coli lysates by means identical to those previously applied to the wild type  $rG_{o\alpha}$ , cross-reacted with rabbit polyclonal anti-bodies against bovine brain  $G_{o\alpha}$  in a manner similar to that of the wild type  $G_{o\alpha}$ . In contrast to the latter, however, the mutants did not serve as substrates for pertussis toxin-catalyzed ADP-ribosylation. These findings are consistent with our earlier demonstration that cysteine 351 is the site of modification by the toxin and with the conclusion that the nature of the amino acid in position 352 (glycine in wild type  $G_{o\alpha}$ ) influences critically the ability of cysteine 351 to serve as an ADP-ribose acceptor.



To begin to define the basis for the apparent multiplicity of  $G_{O\alpha}$  mRNAs that we have found in bovine and human tissues, the  $G_{O\alpha}$  cDNA was used to obtain related clones from a bovine retinal library. Four clones with a Hind III restriction site in the 3' untranslated regions (3' UTRs) that is not present in the original clone were sequenced. The new clones diverge from the original shortly before the Hind III site (35 nucleotides after the stop codon). The 3' UTRs, which are of different lengths, are identical with the exception that two of them contain two extra bases (GT) not present in the others. The coding regions of all clones appear identical, with the exception of a single base in two of the new clones, which would result in the replacement of isoleucine with threonine. There is a threonine in this position in all other reported  $G_{O\alpha}$  sequences.

Based upon differences in this family of cDNAs, selective probes (termed A and B type) for the 3' UTRs were constructed. The full-length original cDNA clone detected three sizes of mRNA in retina, and two in brain. Probe A detected 3.0 and 4.0 kb species in both tissues; it did not hybridize with the 2.0 kb species in retina. The B probes detected the 2.0 and 4.0, but not the 3.0 kb, species. The probes that differentiated between the various mRNAs were used to characterize 55  $G_{O\alpha}$ -like clones from the retinal library. Nine clones failed to hybridize to either type; no clones reacted with both types. There were four A type and 42 B type clones. In all reported  $G_{O\alpha}$  cDNAs (which are from brain), the 3' UTR is of the A type. As the 2.0 kb mRNA was not detected in brain but was abundant in retina, it may well be that the B type transcripts are represented in a retinal library more frequently than in a brain library. Current studies are directed toward understanding the mechanisms responsible for diversity in the 3' UTRs (e.g., alternative splicing) and their tissue-specific expression.

## 2. ADP-ribosylation of Guanine Nucleotide-binding Proteins by Bacterial Toxins: Guanine Nucleotide-binding Proteins that Enhance Choleraen Activity.

Choleraen activates adenylyl cyclase (and thereby causes disease) by catalyzing the transfer of ADP-ribose from NAD to  $G_{S\alpha}$ , the  $\alpha$  subunit of the stimulatory guanine nucleotide-binding protein of the cyclase system. Activation can be enhanced by membrane and/or soluble factors. Last year, we reported purification of three ~ 19 kDa proteins known as ADP-ribosylation factors (ARFs), and presented evidence that they interact with the toxin in a GTP-dependent fashion, enhancing its ability to ADP-ribosylate  $G_{S\alpha}$ , as well as other substrates including simple guanidino compounds (e.g., arginine). It has been shown by others that the ARFs bind guanine nucleotides. Thus, two guanine nucleotide-binding proteins, ARF and ADP-ribosyl  $G_{S\alpha}$ , participate in toxin activation of the cyclase. Detergents can both stimulate and inhibit this process. We find





that ARF stimulation of the NAD:agmatine ADP-ribosyltransferase activity of cholera toxin is enhanced by sodium dodecyl sulfate (SDS) in a concentration-dependent manner. Enhancement of ARF action by SDS is also observed when other toxin-catalyzed reactions are assayed. Conversely, relatively low concentrations of Triton X-100 or CHAPS decrease the magnitude of the ARF effect. Others have reported that SDS promotes toxin activation of adenylyl cyclase, whereas Triton X-100 is inhibitory. In our experiments these detergents, in the concentrations used, did not alter activity of the toxin itself. The observed enhancement or inhibition of ARF stimulation of toxin activity may explain their effects on cyclase activation in membranes.

ARF activity requires GTP (or an analogue) but not dimyristoylphosphatidylcholine (DMPC) and NaCl, which are reported to be necessary for high affinity binding of GTP $\gamma$ S to a membrane ARF. In an attempt to reconcile these observations, we investigated the interaction of guanine nucleotides with a soluble ARF purified from brain. High affinity GTP binding (apparent  $K_D$  ~70 nM) by ARF required  $Mg^{++}$ , DMPC, and sodium cholate, but not NaCl. ARF, in DMPC/cholate, enhanced toxin activity with an apparent  $EC_{50}$  for GTP ~50 nM. There was a delay of ~90 min before a maximal rate of ARF-stimulated activity was attained which was abolished by prior incubation of ARF with GTP at 30°C. In contrast, maximal ARF activation in 0.003% SDS occurred without delay and the apparent  $EC_{50}$  for GTP was ~5  $\mu$ M. High affinity GTP binding by SARF II was not detectable in SDS. Thus, enhancement of toxin ADP-ribosyltransferase activity by ARF can occur under conditions in which SARF exhibits either a relatively low or high affinity for GTP. The interaction of GTP with ARF under these conditions may reflect ways in which intracellular membrane and cytosolic environments could influence GTP-mediated activation of ARF.

To determine whether the effects of ARF and SDS were on the toxin catalytic unit, the active  $A_1$  subunit was prepared by reduction and alkylation. Both SDS and ARF stimulated  $A_1$  activity. Thus, they do not act by promoting or accelerating release of the active  $A_1$  subunit from the holotoxin or the intact A subunit. Our earlier work demonstrated that the kinetics of the cholera toxin-catalyzed reaction are consistent with a rapid equilibrium, random sequential mechanism. Either substrate can bind first to the toxin and in doing so decreases the apparent affinity for the second substrate. A similar kinetic analysis of ARF action shows that ARF decreases apparent  $K_m$ s for NAD and agmatine (the ADP-ribose acceptor) without affecting  $V_{max}$ . In the presence of 0.003% SDS, which itself has little effect, ARF causes larger decreases in the  $K_m$ s and, in addition, apparently increases  $V_{max}$ . These data are consistent with the conclusion that ARF, in the presence of GTP, interacts directly with the toxin  $A_1$  subunit to alter its allosteric properties and SDS enhances this effect.



E. coli heat-labile enterotoxins (LTs), which cause diarrhoeal disease, are structurally similar to cholera toxin and, like that toxin, activate adenyl cyclase by catalyzing the transfer of the ADP-ribose moiety of NAD to a specific arginine in  $G_{sq}$ . Three LTs have been described: LT-I, LT-IIa and LT-IIb. LT-IIa and LT-IIb, which are quite similar to each other, differ considerably in amino acid sequence from cholera toxin and LT-I, which resemble each other. All LTs were activated by ARF in a GTP-dependent manner with maximal enhancement observed in the presence of sodium dodecyl sulfate. Thus, the LT ADP-ribosyltransferases, although they differ from cholera toxin in amino acid sequence, have retained the capacity for ARF stimulation. ARF may play a role in the pathogenesis of disease caused by the LTs as well as in that caused by cholera toxin.

C3T is an ADP-ribosyltransferase produced by type C *Clostridium botulinum* that ADP-ribosylates a 24 kD membrane protein found in many tissues. It has been suggested that the C3T substrate is a GTP-binding protein. Investigating a possible relationship between C3T substrate and membrane ARF, we found that the C3T substrate failed to exhibit ARF-like activity in stimulating auto-ADP-ribosylation of cholera toxin  $A_1$  subunit and ARF did not serve as a substrate for C3T. Thus, although both may be GTP-binding proteins, no functional similarity between ARF and C3T substrate was found.

This year, we initiated cloning and characterization of cDNAs that encode ARF proteins for use in studies defining elements involved in gene structure, expression and function of these proteins. Based on partial peptide sequence of a bovine brain soluble ARF an oligonucleotide probe was constructed to screen a bovine retinal cDNA library. From thirteen positive clones, clone  $\lambda$ ARF2B was selected for sequencing.  $\lambda$ ARF2B contains an open reading frame coding for a protein of 181 amino acids with a calculated molecular mass of 20.7 kDa. Comparison of the deduced amino acid sequence with ARF peptide sequence (total 60 amino acids) revealed only two differences. Whether these resulted from nucleotide substitutions during cloning or reflect the existence of two closely-related, yet distinct proteins is not presently known. Northern blot analysis of retinal poly(A)<sup>+</sup> using the  $\lambda$ ARF2B cDNA as a probe identified only a 2.6 kb species.

Comparison of the deduced amino acid sequences of  $\lambda$ ARF2B, c-Ha-ras p21, and  $G_{O\alpha}$  (see above) revealed similarities apparently limited to regions believed to be involved in guanine nucleotide binding and GTP hydrolysis. The putative domains involved in these functions have been assigned based on models of GDP-binding domains of EF-Tu and the ras p21 protein. Outside of these regions, the amino acid sequences appear quite divergent. For example, the COOH-terminal 40 amino acids of ARF have little similarity with either  $G_{O\alpha}$  or Ha-ras p21. In  $G_{O\alpha}$ , the COOH-

1. The first of these is the fact that the

terminus is thought to be responsible for interaction with receptors. The corresponding region of ras p21 is critical for cell membrane attachment and transformation. ARF lacks a site analogous to those in the G protein  $\alpha$  subunits that are ADP-ribosylated by cholera toxin. Since ARF is also a substrate for the toxin, it appears that in its role as a substrate as well as when acting as an allosteric activator, ARF interacts with cholera toxin in a manner different from the G proteins.

### 3. Characterization of an ADP-ribosylarginine Hydrolase from Turkey Erythrocytes.

We earlier reported the identification in animal cells and subsequent purification of enzymes that, like cholera toxin, exhibit NAD:arginine ADP-ribosyltransferase activity and more recently, the existence of enzymes that can hydrolyze the ADP-ribose-arginine bond. ADP-ribosylarginine hydrolases purified 150,000-250,000-fold (>90%) from the soluble fraction of turkey erythrocytes exhibited one major band (~39 kDa) on sodium dodecyl sulfate-polyacrylamide gels. Mobilities on gel permeation columns were consistent with an active monomeric species of ~39 kDa. Enzyme activity was dependent on dithiothreitol and  $Mg^{2+}$ . Insertion of an organomercurial agarose chromatographic step into the purification procedure resulted in the isolation of a hydrolase exhibiting ~35-fold greater sensitivity to dithiothreitol. Dithiothreitol-sensitive hydrolase was also generated by exposure of the purified resistant enzyme to  $HgCl_2$ . At 20°C, both thiol-sensitive ( $H_S$ ) and thiol-resistant ( $H_R$ ) hydrolases were relatively resistant to N-ethylmaleimide (NEM); incubation with dithiothreitol prior to NEM resulted in complete inactivation.  $Mg^{2+}$  stabilized both  $H_S$  and  $H_R$  against thermal inactivation (with or without thiol). A purified NAD:arginine ADP-ribosyltransferase, in the presence of NAD, inactivated both  $H_S$  and  $H_R$ ;  $Mg^{2+}$  and to a greater extent,  $Mg^{2+}$  plus dithiothreitol, protected both  $H_S$  and  $H_R$  from inactivation. Thus, activation of the hydrolase enhanced its resistance to inactivation by transferase. These observations are consistent with the co-existence in animal cells of active enzymes that catalyze opposing arms of an ADP-ribosylation cycle. The physiological substrates for these enzymes remain to be identified.

### 4. Characterization of the Hepatic $\alpha_2$ -adrenergic Receptor.

The role of the  $\alpha_2$ -adrenergic receptor in liver metabolism has been controversial in regard to the presence of the receptor itself, as well as its precise role in hepatic metabolism. The high degree of proteolysis in hepatic membranes prepared by standard methods coupled with the relatively low affinity for the prototypical  $\alpha_2$  antagonist, yohimbine, have likely prevented clear-cut characterization of the hepatic  $\alpha_2$  receptor. The use of membranes purified on a Percoll gradient and the finding that



rat liver membranes bind the  $\alpha_2$ -adrenergic receptor antagonist, [ $^3\text{H}$ ]-rauwolscine with high affinity ( $K_d$  2-4 nM) permitted us to identify the hepatic  $\alpha_2$  adrenergic receptor population. Competitive binding studies with adrenergic agonists indicate that the affinity for clonidine exceeds that for both epinephrine and norepinephrine. Agonist-binding affinity is reduced in the presence of the GTP analogue, guanylyl- $\beta,\gamma$ , imidodiphosphate. High affinity agonist binding and guanine nucleotide sensitivity are abolished in pertussis toxin-treated membranes. The  $\alpha_2$  adrenergic receptor identified in those studies appears to be of a different sub-type from those reported in platelet and brain preparations. It is the only hepatic receptor thus far shown to be coupled to a pertussis toxin substrate. Accordingly, further characterization of this receptor may provide insight into the role of pertussis toxin-sensitive guanine nucleotide-binding proteins in hepatic metabolism.

#### 5. Regulatory Properties of Cyclic Nucleotide Phosphodiesterases.

Isolated rat fat cells have been used to investigate further the mechanisms of hormonal regulation of the cGMP- and cilostamide-inhibited particulate "low  $K_m$ " cAMP phosphodiesterase. The data suggest that 1) rapid activation of lipolysis and phosphodiesterase by isoproterenol or adenosine deaminase involves increases in cAMP and activation of cAMP-dependent kinase; 2) isoproterenol (or adenosine deaminase) and insulin activate the phosphodiesterase by two distinct mechanisms; 3) the temporal changes in phosphodiesterase activity in the presence of insulin and isoproterenol support the notion that insulin activation does not require elevated cAMP but may be enhanced by elevated cAMP; and 4) the temporal and dose-dependent effects of insulin on activation of phosphodiesterase are consistent with an important role for this enzyme in the antilipolytic action of insulin; and 5) insulin-induced antilipolysis does not absolutely require a functional inhibitory guanine nucleotide-binding protein,  $N_i$ .

In collaborative studies, the particulate low  $k_m$  cAMP phosphodiesterase was purified from bovine omental adipose tissue using cilostamide-agarose as we used last year to purify the enzyme from rat adipose tissue. The catalytic properties of the purified bovine and rat enzymes are very similar. The subunit structure of the bovine enzyme (~77 and 61-63 kDa peptides in SDS-PAGE) differs from the rat. Antibodies raised against the bovine enzyme inhibit activity of, cross-react on Western immunoblots with, and immunoprecipitate both bovine and rat phosphodiesterases.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00622-11 CM

## PERIOD COVERED

October 1, 1987 through September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Cyclic Nucleotide Metabolism

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Joel Moss, M.D., Ph.D. Head, Section on CM, NHLBI  
Mol. Mechanisms

Others: Tristram Bahnson, M.D. Md. Staff Fellow CM, NHLBI  
Barbara Kunz, Ph.D. Visiting Fellow CM, NHLBI  
Patrick Chang Chemist CM, NHLBI  
Sally Stanley Chemist CM, NHLBI  
Martha Vaughan, M.D. Chief CM, NHLBI

## COOPERATING UNITS (if any)

H-C. Chen, Endocrinology and Reproduction Branch, NICHD, NIH  
Randall Holmes, Dept. of Microbiology, USUHS, Bethesda, MD

## LAB/BRANCH

Laboratory of Cellular Metabolism

## SECTION

Molecular Mechanisms

## INSTITUTE AND LOCATION

NHLBI, National Institutes of Health, Bethesda, MD 20892

## TOTAL MAN-YEARS:

3.9

## PROFESSIONAL:

1.9

## OTHER:

2.0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The hormone-sensitive adenylyl cyclase system is the target of bacterial toxins that alter its activity by catalyzing the ADP-ribosylation of critical guanine nucleotide-binding (G) regulatory proteins that couple hormone receptors to the cyclase catalytic unit. An inhibitory G protein (Gi) is ADP-ribosylated by pertussis toxin, an etiological agent in whooping cough, whereas a stimulatory G protein (Gs) is ADP-ribosylated by cholera toxin (CT) and E. coli heat-labile enterotoxins (LT), etiological agents in cholera and "travelers' diarrhea", respectively. The ADP-ribosyltransferase activities of CT and the LTs are enhanced by ~ 19 kDa soluble and membrane guanine nucleotide-binding proteins, known as ADP-ribosylation factors (ARFs); in the case of the LTs, it appears that although two of them, LT-IIa and LT-IIb, diverge in amino acid sequence from LT-I and CT, the sites involved in ARF-stimulated ADP-ribosylation have been conserved. CT and LT catalyze reactions similar to those of ADP-ribosyltransferases endogenous to animal cells. These transferases ADP-ribosylate arginine residues in proteins as well as free arginine. In animal tissues, ADP-ribosylarginine hydrolases exist that cleave the ADP-ribose-arginine linkage, regenerating the arginine residues; these enzymes are active as monomers of 39 kDa; it appears that the ADP-ribosylarginine hydrolase and the ADP-ribosyltransferase could catalyze opposing arms of an ADP-ribosylation cycle analagous to the kinases and phosphatases in protein phosphorylation cyclase. In addition, the ADP-ribosylarginine hydrolases, by reversing the toxin-catalyzed reactions, may promote recovery from disease.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00627-10 CM

## PERIOD COVERED

October 1, 1987 through September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

GTP-binding Proteins and Adenylate Cyclase

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Masatoshi Noda, Ph.D.	Visiting Fellow	CM, NHLBI
Others:	Su-Chen Tsai, Ph.D.	Res. Chemist	CM, NHLBI
	Kim Williamson, Ph.D.	Staff Fellow	CM, NHLBI
	Ronald Adamik	Chemist	CM, NHLBI
	Joel Moss, M.D., Ph.D.	Head, Section on	
		Mol. Mechanisms	CM, NHLBI
	Martha Vaughan, M.D.	Chief	CM, NHLBI

## COOPERATING UNITS (if any)

Klaus Aktories, Rudolf-Buchheim-Institut für Pharmakologie der Justus-Liebig Universität Giessen, West Germany

## LAB/BRANCH

Laboratory of Cellular Metabolism

## SECTION

Metabolic Regulation

## INSTITUTE AND LOCATION

NHLBI, National Institutes of Health, Bethesda, MD 20892

## TOTAL MAN-YEARS:

4.4

## PROFESSIONAL:

3.4

## OTHER:

1.0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Cholera toxin (cholera toxin), the agent responsible for the devastating diarrheal disease characteristic of cholera, causes its effects on cells by activating the hormone-sensitive adenylyl cyclase. Activation results from the ADP-ribosylation of Gsa, a guanine nucleotide-binding regulatory protein of the cyclase complex. The toxin can also ADP-ribosylate other proteins as well as simple guanidino compounds such as agmatine. ADP-ribosylation of Gsa and other substrates is enhanced by so-called ADP-ribosylation factors (ARFs), a family of ~ 19 kDa soluble and membrane guanine nucleotide-binding proteins that have been purified from bovine brain. In the presence of GTP, but not GDP, ARF interacts with the toxin resulting in a decrease in apparent Kms for both NAD and ADP-ribose acceptor with no significant effect on Vmax. Activation of toxin by ARF was increased ~ 4-fold by 0.003% sodium dodecyl sulfate (SDS), which had little or no effect on activity in the absence of ARF; 0.01% SDS abolished toxin activity with or without ARF. In the presence of SDS at stimulatory concentrations, ARF further increased affinities for NAD and agmatine and, in addition, increased Vmax. Stimulatory effects of ARF and SDS were observed in the presence of dithiothreitol with toxin A subunit and in the absence of dithiothreitol, with the reduced and alkylated toxin A1 peptide. Thus, it appears that ARF and SDS do not activate by facilitating release of the toxin catalytic unit. Rather, ARF interacts directly with the catalytic A1 peptide and may thus potentiate the pathogenicity of cholera toxin.

242



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00634-08 CM

## PERIOD COVERED

October 1, 1987 through September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Characterization of cGMP-stimulated Cyclic Nucleotide Phosphodiesterase

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Seiko Murashima, M.D., Ph.D. Visiting Fellow CM, NHLBI

Others: Vincent C. Manganiello, M.D. Head, Section on Bio-chemical Physiology CM, NHLBI  
Ph.D.

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Cellular Metabolism

## SECTION

Biochemical Physiology

## INSTITUTE AND LOCATION

NHLBI, National Institutes of Health, Bethesda, MD 20892

## TOTAL MAN-YEARS:

0.9

## PROFESSIONAL:

0.9

## OTHER:

0.0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

At least three distinct types of cyclic nucleotide phosphodiesterases (PDE) are associated, via different forces, with washed particulate fractions from bovine brain cortex. The detergent-solubilized, particulate cGMP-stimulated PDE was purified via cyclic nucleotide affinity chromatography and heparin-agarose, and exhibits in SDS-PAGE and Western immunoblots an Mr slightly greater than the soluble form of the enzyme isolated from calf liver or bovine brain. The purified particulate PDE is less sensitive to proteolysis by trypsin than the supernatant PDE. Our findings suggest the presence of at least two distinct isoenzymes of the cGMP-stimulated PDE "family" in bovine brain.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00636-07 CM

## PERIOD COVERED

October 1, 1987 through September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Particulate Phosphodiesterase in the Regulation of Lipolysis by Insulin

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Carolyn J. Smith, Ph.D. Staff Fellow CM, NHLBI

Others: Vincent Manganiello, M.D., Ph.D. Head, Sec. CM, NHLBI  
on Biochem.

Marielle Guibbolini, Ph.D. Physiology Visiting Fellow CM, NHLBI

## COOPERATING UNITS (if any)

Eva Degerman and Per Belfrage, Department of Physiological Chemistry, University of Lund, Lund, Sweden; John Egan, National Institute Diabetes, Digestive and Kidney Diseases.

## LAB/BRANCH

Laboratory of Cellular Metabolism

## SECTION

Biochemical Physiology

## INSTITUTE AND LOCATION

NHLBI, National Institutes of Health, Bethesda, MD

## TOTAL MAN-YEARS:

2.3

## PROFESSIONAL:

2.3

## OTHER:

0.0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Isolated rat adipocytes have been utilized to investigate mechanisms of hormonal regulation of the cGMP- and cilostamide-inhibited particulate "low Km" cAMP phosphodiesterase (PDE). Lipolysis, cAMP-dependent protein kinase (A-kinase) and PDE were assayed in the presence of combinations of lipolytic (isoproterenol, ISO; adenosine deaminase, ADA) and antilipolytic effectors (adenosine, Ado; N6-phenylisopropyladenosine, PIA; and insulin).

Incubation of intact adipocytes with either insulin or ISO increases the activity of what may be the same particulate cAMP PDE. Activation by ISO seems to be related to cAMP and A-kinase. Whereas the insulin-induced activation is apparently not dependent upon increases in cAMP, cAMP may enhance the activating effects of insulin. Activation of the particulate PDE by two opposing effectors, i.e., the lipolytic agent ISO and the antilipolytic agent insulin, may ultimately reflect regulation of A-kinase by both hormones. With ISO alone, coordination of adenylate cyclase and PDE may be involved in regulation of cAMP, and hence activity of A-kinase and triglyceride lipase.

Particulate low Km cAMP PDE was purified from bovine omental adipose tissue using cilostamide-agarose; the catalytic properties of the purified bovine enzyme were very similar to the purified rat enzyme. The subunit structure of the bovine enzyme (~77 and 61-63 kDa peptides in SDS-PAGE) differed from the rat. Antibodies raised against the bovine PDE inhibited activity of, cross-reacted on Western immunoblots with, and immunoprecipitated both bovine and rat PDEs.

290





## NOTICE OF INTRAMURAL RESEARCH PROJECT

201 HL 00638-06 CM

## PERIOD COVERED

October 1, 1987 through September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Genes for GTP-binding Proteins

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	S. Russ Price, Ph.D.	Guest Researcher	CM, NHLBI
Others:	James Murtagh, M.D.	Md. Staff Fellow	CM, NHLBI
	C. William Angus, Ph.D.	Staff Fellow	CM, NHLBI
	Maria Nightingale	Chemist	CM, NHLBI
	Joel Moss, M.D., Ph.D.	Head, Sec. Mol. Mech.	CM, NHLBI
	Martha Vaughan, M.D.	Chief	CM, NHLBI

## COOPERATING UNITS (if any)

H.-C. Chen, Endocrinology and Reproduction Research Branch, NICHD

## LAB/BRANCH

Laboratory of Cellular Metabolism

## SECTION

Metabolic Regulation

## INSTITUTE AND LOCATION

NHLBI, National Institutes of Health, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2.9

## PROFESSIONAL:

1.9

## OTHER:

1.0

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Cholera toxin catalyzes the ADP-ribosylation of Gsa, the stimulatory guanine nucleotide-binding protein (GNP) of the adenylyl cyclase system which couples hormone or neurotransmitter receptors to the cyclase catalytic unit. ADP-ribosylation of Gsa increases sensitivity to activation by GTP by inhibiting its intrinsic GTPase, thereby ultimately activating adenylyl cyclase and raising intracellular cyclic AMP.

Recently, one membrane and two soluble guanine nucleotide-binding proteins that enhance the cholera toxin-catalyzed ADP-ribosylation of Gsa have been purified from bovine brain. These ADP-ribosylation factors (ARFs) also serve as toxin substrates. To define the structure and function of the ARFs, we isolated a cDNA clone from a bovine retinal library using a mixed oligonucleotide probe whose sequence was based on the partial amino acid sequence of a bovine brain soluble ARF. There were only two differences between the deduced amino acid sequence of clone  $\lambda$ ARF2B and sequences of two CNBr peptides of the ARF protein (60 amino acids total). Comparison of the deduced amino acid sequences of ARF, Go $\alpha$  (GNP isolated from bovine brain and thought to regulate ion channels), and c-Ha-ras p21 protein revealed similarities in regions putatively involved in guanine nucleotide binding and GTP hydrolysis. ARF lacks a sequence analogous to the site of cholera toxin modification in the  $\alpha$  subunits of the GNPs. We conclude that ARF is a GNP that interacts with the A1 subunit of cholera toxin in a manner quite different from other GNPs. This interaction modifies the catalytic properties of the toxin, in addition to allowing the ARF protein to act as a substrate for ADP-ribosylation.

294



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00639-05 CM

## PERIOD COVERED

October 1, 1987 through September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Characterization of a Bovine Rod Outer Segment Phosphodiesterase

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Seiko Murashima, M.D., Ph.D. Visiting Fellow CM, NHLBI

Others: Vincent C. Manganiello,  
M.D., Ph.D.Head, Section on  
Bio. Physiology

CM, NHLBI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Cellular Metabolism

## SECTION

Biochemical Physiology

## INSTITUTE AND LOCATION

NHLBI, National Institutes of Health, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.1

## PROFESSIONAL:

0.6

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Bovine retinal rod outer segment cGMP PDE and bovine brain particulate and soluble cGMP-stimulated PDEs were purified. After photolabelling with [32P]cGMP, these PDEs exhibit different susceptibilities to proteolytic degradation by Staph V8 protease and trypsin and liberate different major peptides during proteolytic digestion.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00643-02 CM

## PERIOD COVERED

October 1, 1987 through September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Heterologous Expression of Guanyl Nucleotide-Binding Proteins

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: C. William Angus, Ph.D.	Staff Fellow	CM, NHLBI
Others: Suzanne K. Czarnecki, Ph.D.	Staff Fellow	CM, NHLBI
Joel Avigan, Ph.D.	Res. Chemist	CM, NHLBI
Linda Stevens	Chemist	CM, NHLBI
Joel Moss, M.D., Ph.D.	Head, Section on	CM, NHLBI
	Molecular Mechanisms	
Martha Vaughan, M.D.	Chief	CM, NHLBI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Cellular Metabolism

## SECTION

Molecular Mechanisms

## INSTITUTE AND LOCATION

NHLBI, National Institutes of Health, Bethesda, MD 20892

## TOTAL MAN-YEARS:

3.4

## PROFESSIONAL:

2.4

## OTHER:

1.0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Go is one of a family of the guanine nucleotide-binding proteins (G proteins) that are important mediators of signal transduction across biological membranes. Although the precise function of Go is unclear, it appears to be closely associated with calcium flux and receptor-mediated events.

Like the other G proteins, Go is a heterotrimer of  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits. A cDNA clone for the  $\alpha$  subunit of Go was previously isolated and sequenced in this laboratory, and has been used for expression in *E. coli* and in COS cells. *E. coli* expressed the Go $\alpha$  cDNA cloned into the vector pRC-23, with the resulting recombinant protein (rGo $\alpha$ ) being produced at high levels (1-2% of cellular protein). rGo $\alpha$  was immunoreactive with polyclonal antisera raised against bovine brain Go $\alpha$ , and served as substrate for pertussis toxin-catalyzed ADP-ribosylation. The ADP-ribosylation, like that of bovine brain Go $\alpha$ , was enhanced by the presence of  $\beta$  and  $\gamma$  subunits. Furthermore, ADP-ribosylation of rGo $\alpha$  was stimulated by GDP and GTP, but inhibited by non-hydrolyzable GTP analogs, indicating that rGo $\alpha$  contains a functional guanine nucleotide binding site.

Single point mutations were carried out in the cDNA of Go $\alpha$  and two mutants have been expressed in *E. coli*: 1) with cysteine 351 (the site of pertussis toxin catalyzed ADP-ribosylation) replaced by glycine; 2) with glycine 352 replaced by aspartic acid. These recombinant mutant proteins reacted with anti-Go $\alpha$  polyclonal antiserum, but they did not serve as substrates for pertussis toxin-catalyzed ADP-ribosylation.

300



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00644-02 CM

## PERIOD COVERED

October 1, 1987 through September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Characterization of the Hepatic  $\alpha$ 2-adrenergic Receptor

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Stuart I. Harris, M.D., Ph.D. M.D. Staff Fellow CM, NHLBI

Others: Joel Moss, M.D., Ph.D.

Head, Section on CM, NHLBI  
Molecular Mechanisms

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Cellular Metabolism

## SECTION

Molecular Mechanisms

## INSTITUTE AND LOCATION

NHLBI, National Institutes of Health, Bethesda, MD 20892

## TOTAL MAN-YEARS:

0.85

## PROFESSIONAL:

0.85

## OTHER:

0.0

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Studies with isolated liver plasma membranes indicate the presence of a guanine nucleotide-sensitive norepinephrine-binding receptor with low affinity for  $\beta$ -adrenergic and  $\alpha$ 1-adrenergic antagonists. The receptor appears to be of the  $\alpha$ 2-adrenergic subtype based on the observation that it binds the  $\alpha$ 2-selective antagonist rauwolscine with high affinity (2-4 nM). Its affinity for a second  $\alpha$ 2-selective antagonist yohimbine, however, is less than that observed for  $\alpha$ 2-adrenergic receptors in platelet and brain preparations. Competitive binding studies with adrenergic agonists indicate that the affinity for clonidine exceeds that for both epinephrine and norepinephrine. Agonist-binding affinity is reduced in the presence of the GTP analogue, guanylyl- $\beta$ , $\gamma$ , imidodiphosphate. High affinity agonist binding and guanine nucleotide sensitivity are abolished in pertussis toxin-treated membranes indicating that the receptor signal transduction mechanism involves a guanyl nucleotide binding protein.

303





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00645-01 CM

## PERIOD COVERED

October 1, 1987 through September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Tissue-Specific Expression of Guanine Nucleotide-Binding Proteins

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	James Murtagh, M.D.	Md. Staff Fellow	CM, NHLBI
Others:	S. Russ Price, Ph.D.	Guest Researcher	CM, NHLBI
	Eleanor Bruckwick	Chemist	CM, NHLBI
	Joel Moss, M.D., Ph.D.	Head, Sec. on	
		Mol. Mechanisms	CM, NHLBI
	Martha Vaughan, M.D.	Chief	CM, NHLBI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Cellular Metabolism

## SECTION

Molecular Mechanisms

## INSTITUTE AND LOCATION

NHLBI, National Institutes of Health, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2.3

## PROFESSIONAL:

1.3

## OTHER:

1.0

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects    ☒ (b) Human tissues    ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Guanine nucleotide-binding regulatory proteins (GNPs) regulate cell growth and function through the transduction of receptor-initiated signals across cell membranes. Go is a member of the GNP family whose physiologic function has not been defined. Go exists in the brain in high quantities (as much as 1% of membrane protein), and interacts with rhodopsin and muscarinic receptors. Like other known GNPs, Go is a heterotrimer of  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits.

A cDNA clone ( $\lambda$ GO9) containing the complete coding sequence for the  $\alpha$  subunit of the bovine retina Go ( $\text{Go}\alpha$ ) (Van Meurs et al., Proc Natl Acad Sci USA 84:3107, 1987) was used to probe bovine messenger RNA. Three different sizes of  $\text{Go}\alpha$  mRNA were found in bovine retina and two in brain. To determine the molecular basis of the heterogeneity in  $\text{Go}\alpha$  mRNA transcripts,  $\lambda$ GO9 was used as a probe to obtain related cDNAs from a bovine retinal library. Four clones were isolated and sequenced. Their 3' untranslated regions (UTRs) differ markedly from that of the original  $\lambda$ GO9, containing in the divergent area a unique Hind III restriction site not present in  $\lambda$ GO9.

Northern analysis was carried out using 48-base oligonucleotides complementary to unique sequences in the two types of 3'UTRs. The probe complementary to the Hind III site-positive clones detected 2.0 and 4.0 kb  $\text{Go}\alpha$  mRNA species, whereas the probe specific for the 3' UTR of  $\lambda$ GO9 detected only 3.0 and 4.0 kb species. The 2.0 kb mRNA was present in retina, but not in brain. These specific probes to the alternate 3' UTRs should provide tools for studying tissue-specific  $\text{Go}\alpha$  expression.

305



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00646-01 CM

## PERIOD COVERED

October 1, 1987 through September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Characterization of Guanine Nucleotide-binding Proteins

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	David A. Bobak, M.D.	Sr. Staff Fellow	CM, NHLBI
Others:	M. Michael Bliziotes, M.D.	Sr. Staff Fellow	CM, NHLBI
	Kimberly Muczynski, M.D., Ph.D.	Md. Staff Fellow	CM, NHLBI
	Barbara Kunz, Ph.D.	Visiting Fellow	CM, NHLBI
	Joel Moss, M.D., Ph.D.	Head, Sec. on	
		Mol. Mechanisms	CM, NHLBI
	Martha Vaughan, M.D.	Chief	CM, NHLBI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Cellular Metabolism

## SECTION

Molecular Mechanisms

## INSTITUTE AND LOCATION

NHLBI, National Institutes of Health, Bethesda, MD 20892

## TOTAL MAN-YEARS:

3.7

## PROFESSIONAL:

3.7

## OTHER:

0.0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Cholera toxin activates adenylyl cyclase by catalyzing the ADP-ribosylation of Gsa, the stimulatory guanine nucleotide-binding protein of the cyclase system. ADP-ribosylation factor (ARF), was identified as necessary for ADP-ribosylation of Gsa by cholera toxin. ARF, in a GTP-dependent manner, directly enhances cholera toxin ADP-ribosyltransferase activity and auto-ADP-ribosylation of the cholera toxin A1 subunit (CT-A1). ARF activity is markedly enhanced by 0.003% SDS, and to a lesser extent, by 0.1 to 0.5 % sodium cholate. Under these conditions, however, no GTP binding to ARF is detectable.

To determine the basis for this apparent disparity, we investigated, in greater detail, the interaction of guanine nucleotides with ARF. High affinity GTP binding by SARF II (apparent KD of ~ 70 nM) required Mg++, DMPC and sodium cholate. SARF II, in DMPC/cholate, also enhanced CT-A ADP-ribosyltransferase activity with an apparent EC50 for GTP of ~ 5 nM; however, there was a delay of ~ 90 min before achievement of a maximal rate of SARF II-stimulated toxin activity. In contrast, a maximal rate of activation of toxin by SARF II in 0.003% SDS occurred without delay and the apparent EC50 for GTP was ~ 5 µM. Enhancement of cholera toxin ADP-ribosyltransferase activity by ARF, therefore, can occur under conditions in which ARF exhibits either a relatively low or high affinity for GTP. These findings may reflect the way in which subcellular localization could influence the interaction of ARF with GTP and its activation.



Annual Report of the  
Laboratory of Chemical Pharmacology  
National Heart, Lung, and Blood Institute  
October 1, 1987 to September 30, 1988

For many years this Laboratory has been studying possible mechanisms by which drugs and other foreign compounds and their metabolites may evoke various kinds of toxicities. During the past few years most of resources of the laboratory have been devoted to studying various aspects of the mechanisms of immune reactions. One section has directed its attention on mechanisms by which chemically reactive metabolites are synthesized within cells and react with cellular components to form putative antigens which may serve as immunogens or as putative targets in immune mediated mechanisms of cellular toxicity. Another section has directed its attention on the mechanism by which antigens cause the release of histamine and other substances from granules in mast cells and thereby cause allergic reactions. In addition the Laboratory has continued its efforts in studying the mechanisms by which toxicants may decrease intracellular ATP concentrations and subsequently cause cell death. The Laboratory has also initiated studies with cloned isozymes of cytochrome P-450 with model substrates to elucidate factors that govern the substrate specificity and product formation by the individual isozymes.

### Mechanisms of Toxicity

Halogenated anesthetic gases. There is considerable evidence that the fulminant type of hepatitis caused by halothane in humans may be due to an immune reaction. In accord with this view, sera of patients with halothane-induced hepatotoxicity contain antibodies that react to different extents with several hepatic microsomal proteins (100 kDa, 76 kDa, 59 kDa, 57 kDa and 54 kDa) from rabbits and rats treated with halothane. The antibodies are not present in sera of patients who have received halothane but did not manifest hepatitis, nor are they present in sera of patients manifesting other types of hepatic disease. In previous years, we reported that all of the proteins recognized by the antibodies contain trifluoroacetyl groups but that the epitopes must include amino acids adjacent to the amino acids that bind trifluoroacetyl groups. The evidence also indicates that the trifluoroacetyl groups are formed during the oxidative rather than the reductive pathway of halothane metabolism.

The antibodies in the sera of the patients also recognize the 100 kDa and the 76 kDa proteins in liver microsomes from rats treated with enflurane but not those in rats treated with isoflurane or sesame oil. Since enflurane is known to be metabolized to a difluoromethoxydifluoroacetyl halide, the evidence suggests that the antibodies recognize this haptenic group as well as the trifluoroacetyl group. The data also suggest that patients who are sensitive to halothane may also be sensitive to enflurane.

Considerable effort has been made to purify and characterize the various antigens. In the past, the 54 kDa protein was identified as a cytochrome P-450 isozyme inducible by phenobarbital. Moreover for several years, we also believed that the 59 kDa protein was also a cytochrome P-450



isozyme. But end group analysis of the purified 59 kDa protein indicated that it was a previously characterized carboxyl esterase. Indeed the preparation of purified protein not only catalyzed the hydrolysis of p-nitrophenyl acetate, but the turnover number of the enzyme toward this substrate is close to the value reported in the literature.

In collaboration with Dr. Frank Gonzalez (NCI) the Laboratory has raised polyclonal antibodies in rabbits against the purified 59 kDa protein and used the anti 59 kDa antibodies to screen rat liver cDNA libraries constructed in the expression vector  $\lambda$ gt11. Several clones have been isolated and the partial amino acid sequence of the protein assembled. The sequence thus far obtained comprises about 2/3 of the projected length of the 59 kDa protein and includes a region corresponding to the putative action site found in other serine types of esterases.

Studies with the anti-59 kDa antibody preparation have shown that the 59 kDa carboxyl esterase is present predominantly in liver, testes and to a lesser extent in lung and many other tissues. Treatment of rats with dexamethasone decreases the amount of enzyme in liver, testes and fat but did not affect the amount present in lung.

Mechanisms of heme destruction. Many substances, including allylic compounds that cause porphyria and carbon tetrachloride inactivate, cytochrome P-450 by causing the destruction of heme, probably through a free radical mechanism. In a minor pathway of such reactions, however, the heme becomes covalently bound to the protein. In the past we have attempted to elucidate the mechanism by which this covalent binding occurs by studying the covalent binding of heme to cytochrome P-450 54 kDa caused by carbon tetrachloride, but the plethora of reactions occurring in this system makes the interpretation of the results difficult. During the past year, we have switched attention to the reaction between ferrous myoglobin and bromotrichloromethane which also leads to the covalent binding of heme to protein. With this system, it was possible to demonstrate that the heme was bound to a tryptophan located 8 amino acids from the N-terminal end of the myoglobin. In addition the trichloromethyl group reacted with a vinyl group in heme to form 3 adducts which were not covalently bound to the protein. These were a trichloromethyl-heme-carboxylic, a trichloromethyl heme alcohol and a bis trichloromethyl derivative of heme.

Mechanisms of these two kinds of reactions may differ in that the trichloromethyl radical may add directly to heme to form the noncovalently bound heme derivatives but may react with an amino acid in the protein to initiate the formation of the covalently bound heme derivative.

Mechanism of hepatocytes toxicity caused by MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine). This substance causes a Parkinson-like syndrome in humans and primates. There is now considerable evidence that it evokes its toxicity through its conversion by monoamine oxidase B to MPP<sup>+</sup> (1-methyl-4-phenyl pyridinium ion). In an effort to study plausible mechanisms by which MPTP and MPP<sup>+</sup> might cause cell death, some laboratories have used hepatocytes as model cells which contain monoamine oxidases. During the past year our Laboratory has studied the effects of several tetrahydropyridine derivatives and has found that all of the





derivatives that caused cell death were also converted to pyridine derivatives in hepatocytes. Because deprenyl, an inhibitor of monoamine oxidase B, inhibited the formation of the pyridine derivatives to varying extents, however, other enzymes besides monoamine oxidase B, may also be able to form the pyridine derivatives.

Maitotoxin-induced cellular toxicities. Maitotoxin is a polyhydroxy, polyether, disulfate which has a molecular weight of about 3424. In mice it is highly toxic with an LD<sub>50</sub> of about 170 ng/kg. It activates voltage dependent calcium channels in skeletal and cardiac muscle and in certain neural and endocrine cells. In accord with these properties low concentrations kill both hepatocytes (60-80 pM) and cardiac myocytes in culture. Maitotoxin enhances the influx of extracellular Ca<sup>2+</sup>, which leads to a decrease in intracellular ATP and ultimately the release of LDH. The toxicity is decreased if not prevented by lowering the extracellular calcium or by inclusion of verapamil, a voltage dependent Ca<sup>2+</sup> channel blocker.

### Mechanism of Mast Cell Activation and Degranulation

The abundance of mast cells in blood vessels, heart and airways make these sites primary targets for the action of inflammatory mediators that are released from these cells in response to IgE-directed antigens. An understanding of the biochemistry of secretion would assist the development of new therapies for mast cell-mediated allergic reactions. In addition, however, the mast cell and one of its cultured analogs - the RBL-2H3 cell - have proved in general to be versatile models for the study of Ca<sup>2+</sup>-dependent secretion which may be relevant to mechanisms of response in other kinds of cells. Our ability to permeabilize RBL-2H3 cells with full retention of their functional responses and the access to the intracellular environment that this provides has led to the discovery of an array of stimulatory signals, and to the elucidation of mechanisms that modulate these signals. Of particular promise is that such studies have begun to tell us how different signals are orchestrated for secretion.

Antigen-stimulation of RBL-2H3 cells that is triggered by the aggregation of receptors for IgE causes hydrolysis of membrane inositol phospholipids to form inositol 1,4,5-trisphosphate (1,4,5 IP<sub>3</sub>), diacylglycerol (DAG) and other inositol phosphates through a G-protein-dependent activation of a membrane phospholipase C. This early event can be blocked, for example, with neomycin which sequesters polyphosphoinositol lipids or with GDPβS which competes with GTP for binding to G-proteins. This event, in turn, generates a Ca<sup>2+</sup>-signal, through release of bound intracellular Ca<sup>2+</sup> by 1,4,5 IP<sub>3</sub>, and the activation (and translocation) of protein kinase C by DAG. Studies of the amount of 1,4,5 IP<sub>3</sub> in intact cells, the efficacy of exogenous 1,4,5 IP<sub>3</sub> in releasing bound <sup>45</sup>Ca<sup>2+</sup> in permeabilized cells, the pattern of phosphorylation of myosin, and localization of protein kinase C by Western blot techniques provide unambiguous evidence for all of these events in RBL-2H3 cells. We have also shown that the extent of all responses i.e. release of inositol phosphates (phosphoinositide response), calcium signal, and secretion, closely parallel the number of receptors aggregated.



Although intracellular  $\text{Ca}^{2+}$  is mobilized in the intact RBL-2H3 cell, the influx of  $\text{Ca}^{2+}$ , by an as yet undefined mechanism, contributes substantially to the  $\text{Ca}^{2+}$  signal and amplifies the phosphoinositide response. Once initiated, the phosphoinositide response becomes independent of external  $\text{Ca}^{2+}$ . Therefore some undefined  $\text{Ca}^{2+}$ -dependent step, is still required in the early stages of stimulation.

It is commonly assumed that the  $\text{Ca}^{2+}$ -signal and activation of protein kinase C provide synergistic signals for secretion. From studies during the past year, however, it is now apparent that activation of the Na/H antiport by protein kinase C promotes an increase in cytosolic pH ( $\text{pHi}$ ) to generate a pH-signal which supercedes the  $\text{Ca}^{2+}$ -signal, temporally and functionally. At resting  $\text{pHi}$  ( $\sim 7.05$ ), secretion requires a  $[\text{Ca}_i^{2+}]$  of 0.3 to 1.0  $\mu\text{M}$ , i.e. levels reached in stimulated cells. But the  $\text{pHi}$  then increases with time after which the  $\text{Ca}^{2+}$ -dependent secretion requires levels of cytosolic  $\text{Ca}^{2+}$  ( $[\text{Ca}_i^{2+}]$ ) of about 0.1  $\mu\text{M}$ , which is the level in unstimulated RBL-2H3 cells. Studies with pH and  $\text{Ca}^{2+}$ -sensitive probes in intact cells as well as of secretion in permeabilized cells in which  $[\text{Ca}_i^{2+}]$  and the extracellular pH were varied independently thus suggest the following sequence: Initially, the secretory response is totally dependent on an elevated  $[\text{Ca}_i^{2+}]$  but once the  $\text{Ca}^{2+}$  signal subsides (after 3 min) the increasing  $\text{pHi}$  progressively lessens the dependency on  $\text{Ca}^{2+}$ . Secretion is thus maintained until the cell becomes depleted of granules. The process may be economically expedient for the cell because it sustains the response without the need for continuously high, and possibly toxic, concentrations of  $[\text{Ca}_i^{2+}]$ .

Both  $\text{Ca}^{2+}$ -dependent and protein kinase C - dependent processes are certainly brought into play during antigen stimulation. Studies performed in collaboration with Dr. Adelstein indicate that both light and heavy chains of myosin in RBL-2H3 cells are phosphorylated at specific sites by protein kinase C and the light chain is phosphorylated at an additional site by  $\text{Ca}^{2+}$ -calmodulin-dependent light chain myosin kinase. The phosphorylation of the light chain by protein kinase C occurs with both the unphosphorylated form and the form that possesses a phosphate at the myosin kinase site. At the same time, the unphosphorylated species is phosphorylated by the light chain myosin kinase to maintain levels of the monophosphorylated species and even to increase transiently the levels of this species at the onset of secretion. The reverse series of reactions occurs once the secretory response wanes. As the same series of reactions occurs in permeabilized cells, such cells provide the prospect of definitive studies with peptide antagonists and antibodies to these enzymes.

As described in previous years, studies with activators of protein kinase C such as phorbol myristate (PMA) and  $\text{Ca}^{2+}$  ionophores also support the notion of synergistic signals for secretion. Secretion is markedly impaired (80%) when cells are depleted of protein kinase C by prolonged exposure to PMA or by washing the permeabilized cells. The secretory response is not impaired if protein kinase C is translocated into membranes and thereby selectively retained by priming the cells with PMA before permeabilization. This being true, activation of protein kinase C cannot be the only signal that synergies with  $\text{Ca}^{2+}$  in antigen stimulated



cells. Several findings point to alternate synergistic signals. For example, staurosporine, a potent inhibitor ( $K_i$ , 2 nM) of protein kinase C, completely blocks secretion induced by the combination of A23187 and PMA but only partially blocks the antigen-induced secretion at concentrations  $> 20$  nM.

The activation of protein kinase C also serves to down-regulate the early stimulatory events at the level of the receptor or G protein. Down-regulation is not observed under the conditions described above in which protein kinase C is lost. In fact, antigen-desensitized cells can be reactivated by simply permeabilizing the cells and washing them.

The antigen-stimulus is also modulated by stimulatory receptors for adenosine which appear to interact through the stimulatory G-protein ( $G_s$ ) with phospholipase C, independently of adenylate or guanylate cyclase. The receptors have the characteristics of A2 receptors as defined by studies with various adenosine analogs. These agents markedly enhance the stimulatory and secretory responses to antigen and their effects are mimicked in all respects by treatment with cholera toxin which modifies  $G_s$ . Together, cholera toxin and adenosine interfere with each other. Interestingly, antagonists of adenosine receptors block all responses to antigen, in intact and permeabilized cells, even in the absence of adenosine. Endogenous adenosine may thus provide a permissive signal for antigen-induced secretion because adenosine analogs have no action by themselves. Of therapeutic importance, the xanthine antagonists, at submicromolar and micromolar concentrations, block antigen-induced secretion.

### Biochemistry and Kinetics of Drug Metabolism

Factors governing the substrate specificity and product formation of isozymes of cytochrome P-450. Whether a given isozyme of cytochrome P-450 is able to metabolize a given drug to given products depends on several interrelated factors. The substrate specificity depends on the size and shape of the cleft in the isozyme that governs whether the drug is able to gain access to the heme prosthetic group. The rate of metabolism and the pattern of metabolites formed by the isozyme, however, depends on the orientation of the enzyme-substrate complex relative to the heme in the active site, the rigidity of the complex and the energies required to abstract a hydrogen atom from an aliphatic substrate or to form a tetrahedral intermediate between the active oxygen and the  $\pi$  electrons of an aromatic substance.

During the past year we have performed molecular orbital calculations and estimated the heats of reaction for the desaturation and hydroxylation of various substrates. In general, the calculations correspond to the known reactions of the substrates by cytochrome P-450 isozymes. For example, the three reactions known to proceed by dual hydrogen atom abstraction have calculated heats of reaction 4-6 kcal/mol lower than those known to form hydroxylated metabolites by recombination of the radical with the active oxygen radical. Also the relative stabilities of the radical intermediates of 16 compounds appears to correlate with the general relative rates at which the substrates undergo oxidation, i.e. benzylic oxidation,  $\omega-1$  oxidation  $>$   $\omega$ -oxidation.



The results of these theoretical calculations will be tested by studying the metabolism of model substrates by isozymes synthesized from cloned cDNAs inserted into various cell preparations by Dr. Frank Gonzalez and his associates (NCI).

Mechanism of formation of 17 $\beta$ -hydroxy 4,6-androstadiene-3-one ( $\Delta^6$ T).

Isozymes of cytochrome P-450 rarely catalyzed desaturation reactions. The finding by this Laboratory that 6 $\beta$ -hydroxylase and 7 $\alpha$ -hydroxylase convert testosterone to  $\Delta^6$ T thus represented a very unusual event. Studies of the metabolism of various deuterated derivatives of testosterone by reconstituted 7 $\alpha$ -hydroxylase systems during the past year revealed isotope effects that were consistent with the idea that the enzyme initially abstracts the 6 $\alpha$ -hydrogen atom to form a stabilized radical intermediate which then undergoes a 7 $\alpha$ -hydrogen atom abstraction to form the double bond.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00937-06 LCP

## PERIOD COVERED

October 1, 1987 through September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mechanisms of mast cell degranulation: PI breakdown and calcium signal

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Michael A. Beaven

Deputy Chief

LCP

NHLBI

Others: Hydar Ali

Vist. Fellow

LCP

NHLBI

Dolores Collado-Escobar Vist. Assoc.

LCP

NHLBI

## COOPERATING UNITS (if any)

Dr. N.M. Dean, DCT, NCI

## LAB/BRANCH

Laboratory of Chemical Pharmacology

## SECTION

Cellular Pharmacology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20892

## TOTAL MAN-YEARS:

0.6

## PROFESSIONAL:

0.6

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Antigen-mediated stimulation of hydrolysis of inositol phospholipids in RBL-2H3 cells is dependent on the extent and rate of aggregation of the plasma membrane receptors for IgE. The hydrolysis is associated with an increase in concentration of free  $\text{Ca}^{2+}$  [ $\text{Ca}^{2+}$ ]<sub>i</sub>, an increase in cytosolic pH and histamine secretion. Under optimal conditions for stimulation, the rate of generation of inositol polyphosphates, increase in [ $\text{Ca}^{2+}$ ]<sub>i</sub> and increase in cytosol pH reach a maximum at, respectively 15 to 30, 180 and 300 to 500 seconds following addition of antigen. Interestingly, the pH increase becomes most apparent once [ $\text{Ca}^{2+}$ ]<sub>i</sub> begins to decline. This pH signal appears to be generated through activation of protein kinase C and as a consequence of activation of the  $\text{Na}^+/\text{H}^+$  antiport. Accordingly the increase in pH can be reproduced by sole addition of PMA an activator of protein kinase C, but neither PMA nor antigen provoke a pH signal in  $\text{Na}^+$ -free or neutral (pH 7.0) buffers. The pH signal explains previously unexplained effects of PMA which were attributed to a "cryptic signal" in antigen stimulated cells (J.Cell Biol. 105:1129, 1987). Studies with intact, permeabilized and broken cell preparations and variously labeled inositol phosphates have also provided an almost complete map of the pathways of metabolism of the inositol phosphates in RBL-2H3 cells and point to an interesting interconversion of inositol 1,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00962 06 LCP

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Immunological studies on the mechanism of halothane induced hepatotoxicity

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. J. Gerald Kenna Visiting Associate LCP NHLBI

## Other investigators:

Lance R. Pohl Section Chief LCP NHLBI

Hiroko Satoh Visiting Associate LCP NHLBI

Jackie L. Martin Guest Researcher LCP NHLBI

## COOPERATING UNITS (if any)

Brian Martin NSB NIMH

Gene Shearer I NCI

## LAB/BRANCH

Laboratory of Chemical Pharmacology

## SECTION

Pharmacological Chemistry

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda MD 20892

## TOTAL MAN-YEARS:

1.6

## PROFESSIONAL:

1.6

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Our previous research has shown that sera from patients with halothane hepatitis recognize microsomal antigens in rat liver which consist of a metabolite of halothane, the trifluoroacetyl (TFA-) group, bound covalently to specific liver microsomal proteins. We have now found that these protein antigens constitute the major TFA-protein adducts produced in livers of rats exposed to halothane, and that the protein antigens which are the most commonly recognized by the patients' antibodies (100 kDa, 76 kDa, 59 kDa and 57 kDa) are apparently long-lived. Expression of the antigens was increased in rats treated with isoniazid or clofibrate, which induce specific isozymes of cytochromes P-450. This suggests that antigen expression in livers of halothane exposed humans may vary as a function of the hepatic cytochrome P-450 isozyme profile, and that this may be one factor which pre-disposes certain individuals to development of halothane hepatitis. In addition, we found that the antigens can be solubilized from the microsomal membrane using low concentrations of non-denaturing detergents (0.1% deoxycholate or Triton X-100). This property has greatly facilitated our efforts to purify the antigens. Purification to homogeneity is the major goal of the research currently being undertaken. The purified antigens will be used for the detailed biochemical characterization of the proteins, in animal model studies, and as a source of antigen in ELISA methods for the sensitive detection of patients sensitized to halothane or other structurally related inhalation anesthetic.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 HL 00967-06 LCP
PERIOD COVERED October 1, 1987 to September 30, 1988		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Regulation of cytochrome P-450 turnover		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <div style="display: flex; justify-content: space-between; margin-top: 10px;"> <div>P.I. Yoichi Osawa</div> <div>Vist. Fellow</div> <div>LCP</div> <div>NHLBI</div> </div> <div style="margin-top: 10px;"> <div style="display: flex; justify-content: space-between;"> <div>Others: Lance R. Pohl</div> <div>Section Chief</div> <div>LCP</div> <div>NHLBI</div> </div> <div style="display: flex; justify-content: space-between;"> <div>John W. George</div> <div>Chemist</div> <div>LCP</div> <div>NHLBI</div> </div> </div>		
COOPERATING UNITS (if any) Dr. Robert Hyghet, Section Chief LC-NHLBI; Dr. Brian Martin, NSB-NIMH; Dr. Robert Feldman, CR-Dir; Drs. Conney Murphy, Lin Chen, and Robert Cotter, The Johns Hopkins School of Medicine; Drs. John Yates and Donald Hunt, The University of Virginia		
LAB/BRANCH Laboratory of Chemical Pharmacology		
SECTION Pharmacological Chemistry		
INSTITUTE AND LOCATION NHLBI, NIH, Bethesda, Md. 20892		
TOTAL MAN-YEARS:  <div style="text-align: right;">1.7</div>	PROFESSIONAL:  <div style="text-align: right;">1.2</div>	OTHER:  <div style="text-align: right;">0.5</div>
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; margin-top: 5px;"> <div> <input type="checkbox"/> (a) Human subjects  <input type="checkbox"/> (a1) Minors  <input type="checkbox"/> (a2) Interviews         </div> <div> <input type="checkbox"/> (b) Human tissues         </div> <div> <input checked="" type="checkbox"/> (c) Neither         </div> </div>		
SUMMARY OF WORK (Use standard unspaced type. Do not exceed the space provided.) <p>Recently, we have described a novel <u>mechanism-based inactivation</u> of cytochrome P-450 by a variety of drugs that appears to be mediated by radical metabolites and is characterized by the covalent attachment to the protein of products of the heme prosthetic group. In the present study we have investigated the mechanism of the formation of these adducts as well as possible biochemical effects of this modification. A model system for the reaction was developed by reacting ferrous myoglobin with <u>bromotrichloromethane</u>. A major <u>heme-modified protein</u> adduct was produced in the reaction. It was isolated, proteolytically digested, and the resulting principal peptide fragment containing a heme-derived product was characterized. Absorption and mass spectroscopy of the product revealed an intact heme moiety covalently bound to a specific N-terminal tryptophan residue. Three nonprotein bound modified hemes were also isolated from the reaction mixture. These products were characterized as a beta-carboxy substituted vinyl, an alpha-hydroxy-beta-trichloromethyl vinyl, and an alpha,beta-bis-(trichloromethyl)-vinyl derivative of the prosthetic heme by absorption, mass, and NMR spectroscopy. It is believed that the non-protein bound heme-derived products arise from the initial attack of the trichloromethyl radical on a specific <u>vinyl group</u> of heme prosthetic group whereas the covalently bound product likely results from the initial attack of the trichloromethyl radical on the N-terminal tryptophan residue of the protein moiety to create a radical center, which then attacks the heme group. In addition, the heme-modified myoglobin was shown to be more susceptible to trypsin <u>proteolysis</u> than native myoglobin, suggesting a possible role of heme-modification in the protein turnover in vivo of cytochromes P-450 and other heme proteins.</p>		

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00973-04 LCP

## PERIOD COVERED

October 1, 1987 through September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Biochemical mechanisms of mast cell degranulation: Potentiating pathways

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. Dolores Collado-Escobar Vist. Assoc. LCP NHLBI

Others: Michael A. Beaven Chief, Section LCP NHLBI

Heloisa Gonzaga Guest Researcher LCP NHLBI

Hydar Ali Vist. Fellow LCP NHLBI

## COOPERATING UNITS (if any)

Drs. K.P. Huang and F. Huang, ERRL, NICHD

## LAB/BRANCH

Laboratory of Chemical Pharmacology

## SECTION

Cellular Pharmacology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20892

## TOTAL MAN-YEARS:

2.4

## PROFESSIONAL:

2.2

## OTHER:

0.2

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Previous studies have established that antigen-stimulated hydrolysis of inositol phospholipids through the action of phospholipase C initiates a cascade of signals which ultimately lead to secretion in RBL-2H3 cells. Hydrolysis leads not only to the generation of  $\text{Ca}^{2+}$ -mobilizing inositol polyphosphates, but also to the generation of diacylglycerol which causes the activation of protein kinase C. This in turn not only causes the phosphorylation of critical contractile proteins and induces an increase of cytosolic pH which facilitates secretion, but it also modulates the phosphoinositide-response probably at the level of the receptor/G protein complex. The phosphoinositide response, and as a consequence secretion can also be enhanced through activation of stimulatory adenosine receptors - an enhancement mimicked by cholera toxin - possibly through modulation of phospholipase C. This enhancement does not appear to be mediated by cyclic nucleotides or  $\text{Ca}^{2+}$ .





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00975-04 LCP

PERIOD COVERED  
October 1, 1987 through September 30, 1988TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
Signal cascade mechanisms in histamine releasing and nonreleasing RBL clones

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. Hydar Ali Vist Fellow LCP NHLBI

Other:

Michael A. Beaven Deputy Chief LCP NHLBI

COOPERATING UNITS (if any)  
Dr. Joel Moss, LCM, NHLBI and Dr. Reuben Sirganian, Natl. Institute of Dental ResearchLAB/BRANCH  
Laboratory of Chemical PharmacologySECTION  
Cellular PharmacologyINSTITUTE AND LOCATION  
NHLBI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

0.1

PROFESSIONAL:

0.1

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Antigen mediated histamine release from RBL-2H3 cells is associated with substantial hydrolysis of membrane inositol phospholipids and an increase in cytosol  $\text{Ca}^{2+}$  (CA SIGNAL). As reported last year, studies with variants of the RBL-2H3 cells revealed several variants that were completely unresponsive to antigen but did release histamine when challenged with a combination of ionophore and phorbol ester. The possibility of defective coupling between receptor aggregation and activation of phospholipase C was investigated. One such variant BUDR-2B1, showed no or very little phosphoinositide hydrolysis in response to stimulants of GTP-regulatory proteins such as sodium fluoride in intact cells and GTP $\gamma$ S in permeabilized cells. The isolation, purification and hybridization of mRNA with specific radiolabeled probes for two GTP-regulatory proteins, Gi and Go, revealed that the variant and the parental 2H3-cells transcribed message for Go but not for Gi. Another interesting variant showed substantial secretion of histamine and hydrolysis of inositol phospholipids but no  $\text{Ca}^{2+}$  signal as determined with quin 2, Fura 2 and uptake of  $^{45}\text{Ca}^{2+}$ . However, the responses were still dependent on external  $\text{Ca}^{2+}$  and studies with  $^{45}\text{Ca}^{2+}$  revealed that intracellular bound  $\text{Ca}^{2+}$  was released without significant perturbation of free cytosol  $\text{Ca}^{2+}$  levels.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 HL 00983-03 LCP

PERIOD COVERED

October 1, 1987 through September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mechanism of MPTP induced cell death

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Krishnan Kutty Senior Staff Fellow LCP NHLBI

Other: Gopal A. Krishna Chief, Section LCP NHLBI

COOPERATING UNITS (if any)

Dr. Yogendra Singh, USAMRID, Fort Detrick, Frederick, Md.  
Mr. Edward Sokloski, Lab. Chemistry, NHLBI

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Drug Tissue Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20892

TOTAL MAN-YEARS:

0.5

PROFESSIONAL:

0.5

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

It has been previously reported from our laboratory that MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) causes cell death in cultures of rat hepatocytes following its conversion to the toxic metabolite, MPP+ (1-methyl-4-phenyl-pyridinium). The reaction is catalyzed by MAO-B (monoamine oxidase-B) and deprenyl, a specific inhibitor of this enzyme, has been shown to prevent MPTP induced cell death. We have investigated the effect of structural modification of MPTP on the ability to induce cell death. N-acetyl 4'-amino MPTP was found to be virtually nontoxic in cultured hepatocytes, whereas N-butyl PTP, 4'-amino MPTP, and 2'-methyl MPTP were found to be toxic, but to a lesser degree than MPTP itself. The 4'-fluoro and 4'-chloro analogs evoked toxicities similar to that of the parent compound. Treatment of the hepatocytes with deprenyl was found to decrease the toxicities of N-butyl PTP, 4'-amino MPTP, and 4'-chloro MPTP, through the toxicities of the 2'-methyl analog remained largely unaffected. The conversion of all of these compounds, except for the N-acetyl amino analog, into corresponding pyridinium metabolites by liver cells was confirmed by high pressure liquid chromatography and plasma desorption mass spectrometry. Deprenyl was found to inhibit their formation to varying degrees. Moreover, MPTP and its analogs served as substrates for monoamine oxidase in rat liver mitochondria to varying degrees. Even though deprenyl inhibited completely the conversion of MPTP to MPP+, the metabolism by MAO of most of the analogs were inhibited a lesser extent and to varying degrees by deprenyl. These findings indicate that even though the mechanisms of formation of pyridinium metabolites may differ, the formation of the pyridinium metabolites is necessary for the expression of toxicity.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 HL 00984-03 LCP

PERIOD COVERED

October 1, 1987 through September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

A unique testosterone metabolite: 17 $\beta$ -hydroxy 4,6-androstadiene-3-one

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Kenneth Korzekwa

Staff Fellow

LCP

NHLBI

Others: James R. Gillette

Chief

LCP

NHLBI

COOPERATING UNITS (if any)

Drs. Frank Gonzales and Mario Umeno (LMC, NCI)

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Enzyme Drug Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

0.5

PROFESSIONAL:

0.5

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The purpose of this study is to probe the mechanism by which cytochrome P-450a converts testosterone to the metabolite 17 $\beta$ -hydroxy-4,6-androstadiene-3-one ( $\Delta$ 6T). Previous work in this Laboratory, which showed that  $\Delta$ 6T formation paralleled 6 $\beta$ -hydroxylation, suggested that the 6 $\beta$ -hydroxylate catalyzed the reaction and led to the concept of a dual hydrogen atom abstraction mechanism. In addition, however, desaturation reaction is also catalyzed by cytochrome P-450a, which hydroxylates testosterone preferentially to 7 $\alpha$ -hydroxytestosterone. An analogous metabolite of valproate has also been reported. The objective of this work is to measure the deuterium isotope effects associated with the desaturation mechanism. Selectively deuterated testosterone derivatives were synthesized and subjected to metabolism by reconstituted systems of cytochrome P-450a. The isotope effects for  $\Delta$ 6T formation strongly support a stepwise mechanism in which the 6 $\alpha$ -hydrogen atom is initially abstracted, followed 7 $\alpha$ -hydrogen atom abstraction to generate the unsaturated metabolite and water.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00985 03 LCP

## PERIOD COVERED

October 1, 1987 through September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Enzymatic reactions of purified cytochrome P-450 isozymes

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. Henry A. Sasame Chemist LCP NHLBI

Other: James R. Gillette Chief LCP NHLBI

## COOPERATING UNITS (if any)

Dr. Alan Buckpitt, Univ. of California, Davis, CA

Dr. Brian Martin, NIMH

## LAB/BRANCH

Laboratory of Chemical Pharmacology

## SECTION

Enzyme Drug Interaction

## INSTITUTE AND LOCATION

NIH, NHLBI-IR-LCP, Bethesda, Md. 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Naphthalene is metabolized by cytochrome P-450 enzymes to two epoxides, namely, (1S), (2R)-naphthalene oxide and (1R), (2S)-naphthalene oxide. In the presence of glutathione and glutathione transferase mixtures of these epoxides are converted to three glutathione conjugates. Conjugates (1) and (3), which are (1S)-hydroxy-(2S) glutathion-S-yl-1,2,-dihydronaphthalene and (1R)-glutathion-S-yl-(2S)-hydroxy-1,2,-dihydronaphthalene are formed solely from (1S), (2R)-naphthalene oxide. Conjugate (2), which is (1R)-hydroxy-(2R)-glutathion-S-yl-1,2,-dihydronaphthalene is formed solely from (1R), (2S)-naphthalene oxide (Buckpitt, personal communication). Two isozymes of cytochrome P-450 that metabolize naphthalene have been isolated from mouse liver. End group analyses indicate that they are isozymes not previously isolated. Since reconstituted systems containing cytochrome P-450 and glutathione transferases convert naphthalene almost solely to conjugate 2, cytochrome P-450N must oxidize naphthalene almost solely to (1R), (2S)-naphthalene oxide. By contrast, systems containing cytochrome P-450S oxidize naphthalene to both epoxides but preferentially to (1S), (2R)-naphthalene oxide. Studies on the metabolism of naphthalene by microsomes of mouse lung suggest that they contain predominantly cytochrome P-450N. Indeed an antibody against cytochrome P-450(N) almost completely blocked the formation of conjugate 2. By contrast studies with microsomes from mouse liver, suggest that they contain both forms, but mainly cytochrome P-450S. Since naphthalene causes pulmonary damage but not liver necrosis in mice, it may be that (1R), (2S)-naphthalene oxide is more toxic than (1S), (2R)-naphthalene oxide.

340





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00987-02 LCP

## PERIOD COVERED

October 1, 1987 through September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies on the active sites of cytochrome P-450

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. Kenneth Korzekwa Staff Fellow LCP NHLBI

Other: James R. Gillette Chief LCP NHLBI  
Susan Smith Vist. Fellow LCP NHLBI

## COOPERATING UNITS (if any)

Dr. Frank Gonzales and Morio Umeno (LCM, NCI)

## LAB/BRANCH

Laboratory of Chemical Pharmacology

## SECTION

Enzyme Drug Interaction

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20892

## TOTAL MAN-YEARS:

1.5

## PROFESSIONAL:

1.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The cytochrome P-450 enzymes are a family of monooxygenases responsible for the oxidation of a wide variety of endogenous and exogenous compounds. The broad substrate specificities and diverse mechanisms of oxidation of these isozymes makes it difficult to predict the pathways involved in the metabolism of a given compound. It can be rationalized that the regio-specificity of metabolism for a given enzyme-substrate complex is a function of two factors: 1) the orientation(s) of the substrate in the enzymes active site of the isozyme, and 2) the tendency of the substrates functional groups toward oxidation. An understanding of both of these factors for each isozyme together with knowledge of the amounts of each isozyme present, are necessary for predictions in drug metabolism. These studies are in progress to probe these two factors and their interaction: 1) studies of the cytochrome P-450a active sites, 2) theoretical and experimental studies on the oxidation of functional groups and 3) theoretical and experimental studies on the mechanism of aromatase oxidations.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00988-02 LCP

PERIOD COVERED  
October 1, 1987 through September 30, 1988TITLE OF PROJECT (80 characters or less—Title must fit on one line between the borders.)  
Metabolic Basis for Enflurane Hepatotoxicity

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: David D. Christ      Guest Researcher      LCP      NHLBI

## Others:

Lance R. Pohl      Section Chief      LCP      NHLBI

Hiroko Satoh      Vist. Assoc.      LCP      NHLBI

J. Gerald Kenna      Vist. Assoc.      LCP      NHLBI

Jackie L. Martin      Guest Researcher      LCP      NHLBI

## COOPERATING UNITS (if any)

William Kammerer      Anesthesiology Section, Clinical Center, NIH

## LAB/BRANCH

Laboratory of Chemical Pharmacology

## SECTION

Pharmacological Chemistry

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The existence of a rare syndrome of "enflurane hepatitis," similar to that described for halothane and of a cross-sensitization between halothane and enflurane, has been controversial, largely because clinical case reports are equivocal and a plausible molecular mechanism for the hepatotoxicity is lacking. The present study suggests a possible hypersensitivity basis for enflurane hepatitis and the apparent cross-sensitization between halothane and enflurane involving covalently-bound liver microsomal adducts. Immunoblotting studies have revealed that antibodies in the sera of 6 patients with halothane hepatitis recognize liver microsomal antigens of Mr of 100,000, or both 100,000 and 76,000, formed in rats treated with enflurane or halothane. These antigens were not detected in liver microsomes of rats treated with isoflurane or sesame oil. The recognition of these antigens could be abolished by preincubation of the sera with liver microsomes of rats treated with halothane. These data suggest that difluoromethoxydifluoroacetyl halide, a metabolite of enflurane, as well as trifluoroacetyl halide, a metabolite of halothane, covalently bind to similar hepatic proteins, and may become immunogens in susceptible patients. This mechanism may also account for the apparent cross-sensitization between halothane and enflurane anesthesia, and the development of hepatic necrosis.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00989-02 LCP

PERIOD COVERED  
October 1, 1987 through September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Characterization of a novel 59 kDa form of cytochrome P-450

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Hiroko Satoh Vist. Assoc. LCP NHLBI

Others: Lance R. Pohl Section Chief LCP NHLBI  
David D. Christ Guest Research LCP NHLBI  
J. Gerald Kenna Vist. Assoc. LCP NHLBI  
John W. George Chemist LCP NHLBI

COOPERATING UNITS, if any  
Brian Martin, NSB NIMHLAB/BRANCH  
Laboratory of Chemical PharmacologySECTION  
Pharmacological ChemistryINSTITUTE AND LOCATION  
NHLBI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	1.5	PROFESSIONAL:	1.0	OTHER:	0.5
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CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Previous studies have demonstrated the presence of antibodies to novel halothane-induced liver neoantigens in sera from patients with halothane hepatitis, and have suggested that these neoantigens may play an immunopathological role in development of the patients' liver damage. The antibodies have also been shown by immunoblotting to recognize several distinct halothane-induced liver polypeptide neoantigen fractions (100kDa, 76kDa, 59kDa, 57kDa, 54kDa) in liver microsomes that have been covalently modified by the reactive trifluoroacetyl halide (CF33COX) metabolite of halothane. In this paper, the trifluoroacetylated (TFA) 59 kDa neoantigen neoantigen (59 kDa-TFA) was purified from liver microsomes of halothane treated rats by immunoaffinity chromatography with anti-TFA IgG. Antibodies were raised against the 59 kDa-TFA protein and were used to purify the native protein from liver microsomes of untreated rats. Based upon its apparent monomeric molecular weight, NH<sub>2</sub>-terminal amino acid sequence, catalytic activity, and other physical properties, the protein has been identified as a previously characterized microsomal carboxylesterase (EC 3.1.1.1) and not a novel form of cytochrome P-450 as suggested in last years annual report. A similar affinity chromatography approach may be used to purify and characterize the other halothane-induced TFA neoantigens. Immunization of animals with the purified neoantigens, prior to the administration of halothane, will provide an experimental approach for the development of an animal model for this drug hypersensitivity and similar approaches may elucidate other drug hypersensitivities.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00990-02 LCP

## PERIOD COVERED

October 1, 1987 through September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Biochemical mechanisms of mast cell degranulation: Studies with disrupted cell

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I: Hydar Ali

Vist. Fellow

LCP

NHLBI

Others:

Michael A. Beaven

Deputy Chief

LCP

NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Chemical Pharmacology

## SECTION

Cellular Pharmacology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Antigen-mediated exocytosis in RBL-2H3 cells is associated with substantial hydrolysis of membrane inositol phospholipids and an elevation in concentration of cytosol  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]$ ). Studies with intact cells suggested that elevation of cytosol  $\text{Ca}^{2+}$  and activation of protein kinase C provide necessary signals for secretion. A casual relationship between these two events and secretion has now been established in studies with permeabilized RBL-2H3 cells. It is shown that cells appropriately labeled with  $[3\text{H}]$ myoinositol or  $[3\text{H}]-5\text{HT}$  release  $[3\text{H}]$ inositol phosphates or  $[3\text{H}]-5\text{HT}$  when stimulated with antigen or  $\text{GTP}\gamma\text{S}$  at buffered concentrations of free  $\text{Ca}^{2+}$  ( $0.1-1 \mu\text{M}$ ). The phorbol esterase, PMA, which activates protein kinase C, causes release of 5-HT but does not increase the hydrolysis of phosphatidyl inositol. Furthermore release of both  $[3\text{H}]-$ inositol phosphates and  $[3\text{H}]-5\text{HT}$  can be inhibited in parallel by neomycin which sequesters inositol phospholipids and by  $\text{GDP}\beta\text{S}$  which competes with GTP for binding to G-proteins. Another consequence of activation of protein kinase C is the activation of the  $\text{Na}^+/\text{H}^+$  antiport system which in turn elevates the pH of the cytosol in RBL-2H3 cells. The specific roles of  $\text{Ca}^{2+}$ , pH and protein kinase C in the mediation of secretion was studied with permeabilized cells in which it was shown that the requirement for  $\text{Ca}^{2+}$  with either for antigen - or  $\text{GTP}\gamma\text{S}$ -induced secretion can be reduced from  $1 \mu\text{M}$  to  $0.1 \mu\text{M}$  if the pH of the buffer was increased from 7.0 to 7.4. These data suggest that in intact cells secretion can be maintained at low  $[\text{Ca}^{2+}]$  once the intracellular pH of the cells has increased.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00991-02-LCP

## PERIOD COVERED

October 1, 1987 through September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Signal generation and secretion of mediators in rat basophil leukemic cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Theresa N.Lo Research Chemist LCP NHLBI

Others: Michael A. Beaven Deputy Chief LCP NHLBI

Wilford Saul Chemist LCP NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Chemical Pharmacology

## SECTION

Cellular Pharmacology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20892

## TOTAL MAN-YEARS:

1.2

## PROFESSIONAL:

0.4

## OTHER:

0.8

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Ca<sup>2+</sup>-Ionophore-induced secretion of histamine from rat basophil leukemic (RBL-2H3) cell correlated with the hydrolysis of inositol phospholipids but not with the increase in cytosol [Ca(2+)]. However, external [Ca(2+)] was obligatory for secretion (J. Biol. Chem. 262:414,1987). The same pattern was observed for release of arachidonic acid. Similarities were noted in release of histamine and arachidonic acid in antigen-stimulated cells. The release of both were enhanced by treatment with cholera toxin and by the presence of adenosine analogs and were inhibited by the phosphodiesterase inhibitor IBMX in either intact or permeabilized cells. Although their release may depend on the generation of the same signals at the level of the membrane the subsequent mechanisms for release probably diverge: the release of arachidonic acid was 4 to 5 times greater with optimal concentrations of A23187 than with optimal concentrations of antigen whereas the reverse was true for release of histamine. Also inhibitors of protein kinase C or depletion of protein kinase (by prolonged exposure of cells to phorbol ester) resulted in reduced secretion of histamine but enhanced release of arachidonic acid upon stimulation of cells with antigen.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00992-02 LCP

PERIOD COVERED October 1, 1987 through September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
Mechanism of stimulatory and secretory responses of 2H3 cells to  
cardiotoxin

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. Theresa N. Lo Research Chemist LCP NHLBI

Other: Michael A. Beaven Deputy Chief LCP NHLBI

COOPERATING UNITS (if any)  
Dr. C.S. Lo, USUHS, Bethesda, Md.LAB/BRANCH  
Laboratory of Chemical PharmacologySECTION  
Cellular PHarmacologyINSTITUTE AND LOCATION  
NHLBI, NIH, Bethesda, Md. 20892

TOTAL MAN-YEARS: 0.3

PROFESSIONAL: 0.3

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

Cultured rat basophilic leukemia (RBL-2H3) cells labeled with myo[2-<sup>3</sup>H]inositol for 24 hr showed, upon exposure to cobra cardiotoxin (Maja maja kaauthia), a substantial (5 to 7 fold) increase in the amount of <sup>3</sup>H-label in the phosphatidyl inositol monophosphate (PIP) pool. The extent of increase was dependent on concentration and time of exposure to cardiotoxin. The kinetics of redistribution of label within the various inositol lipid and inositol phosphate pools suggested that a major action was the stimulation of phosphatidylinositol kinase activity which converts phosphatidylinositol (PI) to PIP. This action was confirmed by assay of the enzyme activity in membrane preparations. Cardiotoxin also caused a time- and concentration-dependent increase in inositol phosphates (4-8%), a slow increase in cytosol Ca<sup>2+</sup>, secretion of histamine and release of lactate dehydrogenase (LDH) from the 2H3 cells. The release of histamine but not LDH was totally dependent on external calcium. The results suggest that cardiotoxin-stimulated release of histamine from 2H3 cells is through a Ca(2+)-dependent noncytotoxic process. This and previous studies indicate the presence of highly active inositol phospholipid kinases and phosphomonoesterases in membranes of RBL-2H3 cells.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00993-02 LCP

## PERIOD COVERED

October 1, 1987 through September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Phosphorylation of myosin heavy and light chains in stimulated basophils

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Russell Ludowyke

Vist. Assoc.

LCP

NHLBI

Other:

Michael A. Beaven

Deputy Chief

LCP

NHLBI

## COOPERATING UNITS (if any)

Drs. Robert S. Adelstein and Itzhak Peleg, LMC, NHLBI

## LAB/BRANCH

Laboratory of Chemical Pharmacology

## SECTION

Cellular Pharmacology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 220892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A rat basophil leukemic cell line (RBL-2H3) exhibited phosphorylation of both the 200,000 and 20,000-dalton chains of myosin following antigenic stimulation. Cells were primed with antigen specific IgE and labeled with [32P] orthophosphate and [35S]-methionine and then stimulated with antigen. Cells were then disrupted and myosin was isolated by immunoprecipitation with anti-platelet myosin antibodies. Upon stimulation, the amount of [32P]phosphate incorporated into the myosin chains increased from 0.4 to 0.8 moles/mole of light chain of myosin and from 0.8 to 1.6 moles/mole of the heavy chain of myosin. Most of this additional phosphorylation was accounted for by the incorporation of the phosphates into sites on both chains that are known to be phosphorylated by protein kinase C, although at the onset of antigen-induced secretion a transient increase in phosphorylation was observed at a site targeted by calmodulin-dependent light chain myosin kinase. The phosphorylation of the protein kinase C sites was more sustained. The subsequent dephosphorylation of these sites (after 10 to 15 min) occurred as the secretory response subsided. Additional studies revealed that both unphosphorylated and monophosphorylated forms of the light chain of myosin were phosphorylated by protein kinase C. The unphosphorylated form was also phosphorylated by the myosin kinase and the levels of the monophosphorylated form were thereby maintained at constant levels. The net effect was the de novo generation of the diphosphorylated form of the



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00995-01 LCP

## PERIOD COVERED

October 1, 1987 through September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mechanism of maitotoxin-induced toxicity

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Krishnan Kutty Senior Staff Fellow LCP NHLBI

Other: Gopal A. Krishna Chief, Section LCP NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Chemical Pharmacology

## SECTION

Drug Tissue Interaction

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20892

TOTAL MAN-YEARS:

0.5

PROFESSIONAL:

0.5

OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Maitotoxin, a highly potent marine toxin (minimum lethal dose of 170 ng/kg, i.p., in mice) isolated by Japanese workers from Gambierdiscus toxicus has been shown to activate voltage dependent calcium channels in skeletal and cardiac muscle as well as certain neuronal and endocrine cells. Maitotoxin has been shown to be cardiotoxic. In the present study we have investigated the mechanism of cell death in cultured rat hepatocytes. Cell death was monitored by leakage of lactate dehydrogenase (LDH). Maitotoxin induced a time as well as dose dependent cell death with a LD50 of 60-80 pM at 24 h. The cell killing by maitotoxin was completely abolished by omission of calcium from the culture medium. Cell death induced by maitotoxin was increased by increasing calcium from 30  $\mu$ M to 3000  $\mu$ M. Maitotoxin markedly increased influx of calcium within minutes as shown by uptake of  $^{45}\text{Ca}$ . The uptake of  $^{45}\text{Ca}$  was both time and dose-dependent. However, higher concentrations of maitotoxin caused a rapid uptake followed by leakage of  $^{45}\text{Ca}$  which paralleled the leakage of LDH. Verapamil, a specific calcium channel blocker, completely blocked the toxic effect and the  $^{45}\text{Ca}$  influx of lower concentrations of maitotoxin and partially blocked the toxicity induced by the higher concentrations of maitotoxin. Maitotoxin induced cell death is due to a marked reduction in cell ATP since it caused a time and dose-dependent loss of cell ATP. Loss of cell ATP occurred following rapid calcium flux. Since calcium is known to interfere with the ability of mitochondria to synthesize ATP, we postulate that the cell killing action of maitotoxin is due to the inhibitory effect of calcium on ATP synthesis.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 0J996-01 LCP

## PERIOD COVERED

October 30, 1987 through September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mechanism of maitotoxin-induced cardiotoxicity

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Giovanni Santostasi      Guest Researcher      LCP      NHLBI

Others: Krishnan Kutty      Senior Staff Fellow      LCP      NHLBI

Gopal A. Krishna      Chief, Section      LCP      NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Chemical Pharmacology

## SECTION

Drug Tissue Interaction

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20892

## TOTAL MAN-YEARS:

0.5

## PROFESSIONAL:

0.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

Maitotoxin, a highly potent marine toxin isolated from dinoflagellate, *Gambierdiscus toxicus*, has been shown to activate voltage dependent calcium channels. It has also been shown to be cardiotoxic. The mechanism is not known. In the present study we have investigated the mechanism of maitotoxin induced cell death in neonatal rat cardiomyocytes maintained in culture. Maitotoxin induced a time and dose dependent cell killing of cardiac myocytes. Cell death was monitored by leakage of the cytoplasmic enzyme lactate dehydrogenase. Maitotoxin induced cell death was dependent on the presence of millimolar concentrations of calcium in the medium. Reduction of extracellular calcium to 100  $\mu$ M markedly reduced the cell killing effect of maitotoxin. Maitotoxin markedly increased the influx of calcium in a dose dependent fashion. It caused a rapid flux of calcium into the cells which after reaching a peak declined. This reduction was due to cell death as shown by leakage of LDH at this time. The uptake of calcium induced by maitotoxin was blocked by the calcium channel blocker, verapamil.  $Mn^{2+}$ , however, was much less effective at equimolar concentration (100  $\mu$ M). Both of these blockers also inhibited maitotoxin induced cell death as measured by LDH leakage. The mechanism of cell death induced by maitotoxin appears to be related to its capacity to lower cell ATP. Lowering of cell ATP was routinely monitored by leakage of ( $^{14}C$ )-adenine nucleotides from cells which has been prelabeled with ( $^{14}C$ )-adenine. The leakage of adenine nucleotides occurs before leakage of LDH, but only after increased influx of calcium. The primary effect of maitotoxin on cardiotoxicity thus appears to be an effect on calcium flux. The increased calcium within the cell inhibits and/or uncouples oxidative phosphorylation resulting in a marked reduction in cell ATP which determines cell death.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00997-01 LCP

PERIOD COVERED  
October 1, 1987 to September 30, 1988TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
Regulation of Microsomal Carboxylesterases by Glucocorticoids

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: David D. Christ

Guest Researcher

LCP NHLBI

Other Investigators:

Lance R. Pohl

Section Chief

LCP NHLBI

Hiroko Satoh

Visiting Assoc.

LCP NHLBI

Rochelle M. Long

Staff Fellow

LCP NHLBI

COOPERATING UNITS (if any)

Brian Martin, NSB, NIMH

LAB/BRANCH  
Laboratory of Chemical PharmacologySECTION  
Pharmacological ChemistryINSTITUTE AND LOCATION  
NHLBI, NIH, Bethesda MD 20892

TOTAL MAN-YEARS:

0.7

PROFESSIONAL:

0.7

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Microsomal carboxylesterases are a family of enzymes that hydrolyse the ester and amide bonds of xenobiotics and are also thought to have a role in the metabolism of endogenous fatty acid esters. The precise function and regulation of these enzymes, however, is not well understood. In the present study, we have investigated the tissue distribution and regulation by corticosteroids of a 59 kDa form of these enzymes, which we have recently purified and characterized from rat liver microsomes.

Immunoblot analysis of several tissues from rats, revealed that the enzyme is present predominantly in the liver, testes, and adipose tissue, and was decreased 3 to 6 fold in each of these tissues after treatment of rats with the corticosteroid, dexamethasone. The amount of the 59 kDa protein in the lung did not appear to be affected by treatment with dexamethasone. These findings show that the 59 kDa enzyme is present in many tissues of the body. They also indicate that corticosteroids may serve as an important pharmacological tool for elucidating the physiological function of the 59 kDa protein in each of these tissues.

571



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00998 01 LCP

## PERIOD COVERED

October 1, 1987 - September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular biology of a 59 kDa carboxylesterase

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Rochelle M. Long Staff Fellow LCP NHLBI

## Other Investigators:

Lance R. Pohl

Section Chief LCP NHLBI

Hiroko Satoh

Visit. Assoc. LCP NHLBI

## COOPERATING UNITS (if any)

Frank J. Gonzalez and Shioko Kimura, Lab. Molecular Carcinogenesis, NCI;  
Brian Martin, NSB, NIMH

## LAB/BRANCH

Laboratory of Chemical Pharmacology

## SECTION

Pharmacological Chemistry

## INSTITUTE AND LOCATION

NHLBI, NIH, BETHESDA, MD 20892

## TOTAL MAN-YEARS:

1.1

## PROFESSIONAL:

1.1

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

It was recently discovered that one of the immunogens associated with halothane-induced hepatitis is a trifluoroacetylated form of a 59 kDa liver microsomal carboxylesterase. In order to learn more about the structure, regulation, and physiological function of this enzyme and its role in halothane hepatitis, a study of its molecular biology has been initiated. Polyclonal anti-59 kDa antibodies raised in a rabbit was used to screen rat liver cDNA libraries constructed in the expression vector lambda gt11. Several positive clones were isolated, subcloned, and sequenced by double-stranded and shotgun cloning techniques in conjunction with dideoxy sequencing methodology. A partial sequence was assembled based upon these results and sequence information derived from tryptic peptides of the native 59 kDa protein that corresponded to approximately 2/3 of the projected length of functional 59 kDa protein. This sequence contained the putative active site regions found in other serine-type esterases. Further analysis of other clones is ongoing in order to yield complete protein and genetic data for the rat liver 59 kDa microsomal carboxylesterase and its possible isozymes. The clones will serve as genetic probes for the investigation of the tissue distribution and regulation of this enzyme and ultimately will help to elucidate its physiological function and role in halothane hepatitis.

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ANNUAL REPORT OF THE  
LABORATORY OF CHEMISTRY  
SECTIONS ON CHEMICAL STRUCTURE AND STRUCTURAL  
NUCLEAR MAGNETIC RESONANCE  
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

October 1, 1987, through September 30, 1988

Nuclear Magnetic Resonance is a major activity of the laboratory under Drs. Ferretti and Highet. The spectrometers are all operating satisfactorily, providing useful data.

J. Ferretti's group is still involved in AIDS related peptides where two fragments have been synthesized. One of these is the fragment with the highest hydrophobic moment found in the envelope glycoprotein and thus thought to be an immunodominant T-cell site. Its conformation has been partly determined as containing both alpha- & beta-turn regions.

The group has also studied binding of the 1-13 fragments of ACTH strands in terms of the known hydrophilic-hydrophobic peptide codon relationship. Somewhat surprisingly, no evidence for binding was found, but some binding in methanol may occur when the chain is extended to 24 amino acids. ;

Using a complex series of nmr pulse methods and other physical measurements on a series of closely related tachykinins, Ferretti's group has been able to get complete internuclear distances and thus conformations. The results show that the peptides have little ordered structure in water but can be described by a set of rapidly interconverting beta-type turn structures in methanol. The biologically active terminal carboxamido linkage is involved intramolecularly with Gly-9 and this may serve as a recognition site for receptor binding.

His group has further completely assigned the proton resonances of the B loops of human TGF-alpha and EGF. NMR distance constraints and molecular dynamics calculations, including solvent effects were used for the first time to calculate energy minima revealing significant differences in the turn regions of each peptide, and this effect has been related to antigenicity. Thus monoclonal antibodies raised against EGF recognize the B loop but those raised against TGF-alpha do not recognize the corresponding B loop. Since neither loop was capable of displacing EGF from its receptor, the major antigenic determinant found in the EGF B loop is not sufficient for receptor binding. Finally, the Ferretti group continues to study sources of error in NMR spectra determination. This has recently led to a technique to determine optimal apodization functions.

Using NMR, R. Highet has established the structures of a bromotrichloromethone metabolites of myoglobin, showing the vinyl group of the heme fragment to have added CO<sub>2</sub>, HOCCl<sub>3</sub> and 2 CCl<sub>2</sub> elements. Using the same method, he has also solved the structures of several of the toxic glutathione adducts of bromo-hydroquinone, a new homologue of the alkaloid cassine, and studied the failure of phloroglucinol to convert to the trione in trichloroethanol.

T. Jones is developing syntheses of naturally occurring 3,5-dialkylpyrrolizidines and dialkylindolizidines found in insect venoms. He has also made progress in





the synthesis of the alkaloid castanospermine, a compound active in vitro against the AIDS virus. In all of these syntheses, stereoselectivity is the key issue and new methods must be developed to invoke this feature in the construction of 2,5-substituted 5-membered rings and their fused pyrrolizidine analogs.

E. Sokoloski has applied the PDMS mass spectral method to the analysis of metabolites of MPTP which produces symptoms of Parkinsonism in humans. The active intermediate is believed to be a quaternary ammonium compound and thus ideally suited for analysis by this method (with G. Krishna, CP-NHLBI). He and Fales have also elucidated the structure of an oligosaccharide from oral microbes (S. Sanguis, F. Cassels, NDI) using PDMS as Rh-Rh-Glu-Gal-Gal-Gal.

J. Silverton has pursued molecular mechanics methods of establishing energy minima for structures in solution by using data from NMR and x-ray crystallography. Fairly good agreement was obtained in the case of an actinomycin and, in the case of medium rings and two AIDS drugs, it was established that energy barriers between several conformations was small. He has spent considerable effort this year in implementing and improving an updated version of the XTAL crystallographic programs. Several rate limiting steps in the program have been greatly improved. Besides completing structure of acenaphthol, a steroid oxidase inhibitor, and rubescensin B, Silverton has initiated studies on isoguanosine, and a theoretical examination on the existence of the pseudo-crystalline state of matter. These substances, while not crystalline, as that term is defined, are ordered and so diffract x-rays.

H. Lloyd continues her studies on physiologically active plant and insect substances. Brunfelsia grandiflora clearly has other substances besides pyrrolle-3-carboxamide that are physiologically active and two fractions inducing reactions in mice are being studied. She has isolated isomultiflorenol acetate, a new compound in nature from the waxy covering of benincasa hispida and several other related substances are also being studied. Several new insect products have been discovered from Myrmecaria, L. meridionalis and the Pentatomidae family. One of these is a new monoterpene whose structure proof involved a complex relationship to its mass spectrum, emphasizing the need for new approaches to the latter technique.

H. Fales and R. Mason have elucidated the structures of a complex series of ketoesters from the wax beetle, and also discovered the sex pheromone of the common garter snake to be a series of long chain saturated and unsaturated 2-methyl ketoses. Fales has used the PDMS system to solve a wide variety of problems including peptide molecular weight determinations, glycosyl conjugates, distributions of oligomers in cyclodextrin, and a new complex oligosaccharide from an oral bacteria.

Using GC-MS he has also elucidated the structure of 16 new pyrazines from a New Zealand ant, found o-aminoacetophenone to be a bee repellent, and characterized a series of epoxidized sterols implicated in prostatic cancer.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01002-14 CH

## PERIOD COVERED

October 1, 1987, to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Application of Nuclear Magnetic Resonance to Biochemical Systems

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Edward A. Sokoloski

Chemist

CH NHLBI

## COOPERATING UNITS (if any)

Dr. Gopal Krishna, NHLBI:CP

## LAB/BRANCH

Laboratory of Chemistry

## SECTION

Nuclear Magnetic Resonance

## INSTITUTE AND LOCATION

NIH:NHLBI, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Cf-252 Plasma desorption mass spectroscopy study of 1 Methyl-4-Phenyl-1,2,3,6 tetrahydropyridine and analog metabolites. Toxicity is found to correspond with formation of 1-Methyl-4Phenyl-Pyridinium ion. Cf-252 PDMS and Nuclear Magnetic Resonance study of hexasaccharide isolated from Streptococcus Sangius.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01003 16 CH

## PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structure of Natural Products Using Instrumental Methods

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. H.M. Fales, Ph.D Chief, Laboratory of Chemistry, NHLBI, CH  
OTHER R. Mason, Ph.D. Staff Fellow

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemistry

SECTION

Chemical Structure Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

2.0

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

A variety of natural products have been studied using GC-MS, NMR and HPLC including a series of lipids and other products from insects. The Cf-PDMS system has been successfully applied to peptides, glucosyl conjugates, cyclodextrins, and polysaccharides.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01004-17 CH

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Characterization of Natural Products

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: H. A. Lloyd Research Chemist CH NHLBI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Chemistry

## SECTION

Chemical Structure

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

## TOTAL MAN-YEARS:

1

## PROFESSIONAL:

1

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The work involves the structure determination of physiologically active compounds of plant and animal origin. Various types of chromatography (gas, thin layer, ion exchange, liquid) are used to isolate pure samples of unknowns. Structures are determined by chemical methods (degradation and synthesis) and with the aid of spectrometry (infra-red, UV, NMR and mass spectrometry).

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01005-17 CH

## PERIOD COVERED

October 1, 1987, to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Solid State and Computer Studies of Physiologically-Important Molecules.

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

J. V. Silverton

Research Chemist

CH NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemistry

SECTION

Chemical Structure

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:

1.0

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Solid State and computational work of the Laboratory of Chemistry, NHLBI concerns Structural Conformational and configurational studies of biologically-interesting compounds. Compounds with enzymatic action, therapeutic value (AIDS drugs) and natural compounds and drugs have been investigated.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 HL 01006-17 CH
PERIOD COVERED October 1, 1987, through September 30, 1988		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) The Characterization of Natural Materials and Metabolic Products		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
P.I.     Robert J. Highet OTHER:   I. V. Ekható, Ph.D.	Research Chemist Visiting Fellow	CH NHLBI CH NHLBI
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Chemistry		
SECTION Structural Nuclear Magnetic Resonance Section		
INSTITUTE AND LOCATION NHLBI, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS: 2	PROFESSIONAL: 2	OTHER:
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  NMR Studies have established the structures of modified hemins from myoglobin and of toxic thiohydroquinones. Phloroglucinol has been shown to form a tris sodium bisulfite addition compound. A study of the alkaloids of <u>cassia excelsa</u> has been resumed.		

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 HL 01027-06 CH

PERIOD COVERED

October 1, 1987, to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Nuclear Magnetic Resonance Spectroscopy on Biologically Important Molecules

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

James A. Ferretti, PhD	Research Chemist	NHLBI CH
Susan Sumner, PhD	Staff Fellow	NHLBI CH
Kyou-Hoon Han, PhD	Visiting Scientist	NHLBI CH

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemistry

SECTION

Structural Nuclear Magnetic Resonance Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:

3.0

PROFESSIONAL:

3.0

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Research involves the development and application of multiple pulse Fourier transform methods in nuclear magnetic resonance spectroscopy, including solvent suppression and two dimensional techniques. Applications include conformational properties of peptides and small proteins in solution, studies in the precision of the methodology, and physical properties of peptide-peptide complexes



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01029-01 CH

## PERIOD COVERED

October 1, 1987 - September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Synthesis of Saturated Nitrogen Heterocycles

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. Tappey Jones Senior Staff Fellow:NHLBI:CH

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Chemistry

## SECTION

Chemical Structure Section

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1

## PROFESSIONAL:

1

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Research was directed at the synthesis of saturated nitrogen heterocycles. The goals of this work are the development of new preparative methodologies and the elucidation of structure in cases where insufficient natural material is present to make this possible by the usual spectrometric means.





Annual Report of the Clinical Hematology Branch  
National Heart, Lung and Blood Institute  
October 1, 1987 to September 30, 1988

The research of this Branch is directed toward understanding the underlying causes and developing effective treatment for major hematological disorders, primarily those affecting the red cell. Red cell disorders that produce significant morbidity and mortality include thalassemia, severe hemoglobinopathies of which sickle cell anemia is the most common, and the various syndromes of bone marrow failure. The molecular mechanisms of globin gene regulation during development are being defined and new strategies to manipulate HbF synthesis are under investigation. A major goal is the development of genetic therapy for hemoglobin disorders. Strategies are being devised and tested to introduce expressing globin genes into bone marrow cells in vivo. The role of various growth factors and their receptors in normal hematopoiesis and in leukemogenesis is under investigation. The use of anti-sense nucleic acid sequences to modulate gene expression is being explored as a tool for study of gene function and potentially for therapeutic intervention-particularly in acquired immunodeficiency syndrome. The pathogenesis and treatment of aplastic anemia and the role of viruses in bone marrow failure are undergoing intense investigation.

Patients with either severe beta-thalassemia or sickle cell anemia could benefit from increased production of fetal hemoglobin. Fetal hemoglobin (HbF= $\alpha_2\gamma_2$ ) produced in utero, is rapidly replaced during the perinatal period with the adult type of hemoglobin (HbA= $\alpha_2\beta_2$ ). At the gene level, this switch reflects turn off of the gamma globin genes and turn on of the beta globin genes. If both beta genes are defective, the switch leads to the onset of hematological disease.

Regulation of the switch involves the interaction of trans-acting factors (proteins) with control (cis-active) DNA sequences linked to the globin genes. Previous work in the laboratory has identified several cis-active sequences that are important in the modulation of gene expression during development (Individual projects: "Regulation of hemoglobin switching during development: characterization of globin gene promoters" and "Identification of cis and trans-acting elements that regulate human gamma gene expression"). We have recently obtained evidence for several specific binding sites within the gamma globin promoter region (Individual project: "DNA binding proteins that interact with gamma gene promoters"). A single complex of proteins appears to bind, with sequence specificity, to two positions separated by 120 base pairs of DNA. The binding of one of these is markedly reduced by a point mutation in DNA that, in vivo, is associated with hereditary persistence of fetal hemoglobin. These data suggest that this particular protein may function as a repressor. Partial purification of these proteins has been achieved with ion exchange chromatography; further efforts at purification will utilize affinity columns with specific DNA oligonucleotides.



Synthesis of fetal hemoglobin can be induced in adults with several chemotherapeutic agents. We have shown that the hematopoietic growth factors, interleukin 3 and erythropoietin, interact to augment production of red cells containing fetal hemoglobin in non-human primates. Further, erythropoietin interacts with hydroxyurea in markedly augmenting the levels of HbF that can be achieved by the chemotherapeutic agent (Individual project: "Pharmacological manipulation of HbF synthesis"). In collaborative clinical studies we have shown that chronic hydroxyurea treatment increases HbF in approximately 60% of patients with sickle cell anemia. The magnitude of increase is substantial although at the expense of significant hematological toxicity. Our intent is to develop protocols utilizing erythropoietin and hydroxyurea simultaneously, based on the encouraging data that we have obtained in animal trials.

Genetic therapy for severe beta thalassemia involving gene replacement is a goal of our research effort. Retroviral vectors containing the human beta globin have been shown to transfer that gene with high efficiency into multipotential hematopoietic progenitors of mice. Expression occurs in the progeny of virtually all progenitors in which the retrovirally transferred globin gene has been integrated (Individual project: "Use of viral regulatory sequences to facilitate gene transfer and analysis of gene function"). Two problems remain to be solved. The frequency of infection of repopulating stem cells is low with currently utilized protocols. We are developing experimental conditions that utilize purified recombinant hematopoietic growth factors to manipulate the cell cycling characteristics of stem cells and to maintain these cells in culture. Preliminary data suggest this approach may markedly enhance the efficiency of stem cell infection. The second problem is the relatively low level of globin gene expression in hematopoietic cells in vivo. Additional cis-active DNA segments that function as regulatory elements will be incorporated into the retroviral vectors in an effort to achieve a design that ensures a high level of gene expression. Modification of the surface of the retroviral particles to target to specific cells may enhance the potential of such vectors for genetic therapy (Individual project: "Modification of retroviral targeting by hybrid envelope proteins"). Engineering of chimeric proteins that retain essential features of both the ligand and the retroviral envelope protein has proved challenging. New information regarding the mechanism of retroviral entry into cells has suggested alternative designs that are currently being pursued.

Molecular characterization of hematopoietic growth factors and their receptors has opened many new avenues of investigation into the mechanisms of hematopoiesis. During the past year, we have molecularly cloned the cDNA coding sequences for the mouse c-fms gene product (Individual project: "Structure and function of the murine M-CSF receptor"). It functions as the receptor for macrophage-colony stimulating factor (M-CSF). In vitro mutagenesis of these coding sequences is being pursued in an effort to define the structural features required for signal transduction. Specifically, the role of the trans-membrane segment and the relevance of allosteric changes in the catalytic domain upon ligand binding are under investigation. A



chimeric construction between the human EGF receptor ligand binding domain and the human c-fms cytoplasmic catalytic domain has been constructed. This protein is synthesized normally and undergoes autophosphorylation on addition of EGF but to date has not transmitted a mitogenic stimulus in cells bearing the chimeric receptor (Individual project: "Function of proto-oncogenes in human hematopoietic cells").

The 5q- syndrome is a refractory anemia characterized by the presence of an abnormal chromosome 5 in bone marrow cells. An interstitial deletion of the long arm of 5 has removed the genes for several hematopoietic growth factors and receptors including IL-3, GM-CSF, M-CSF and the M-CSF receptor. Speculation has focused on whether hemizygoty for these genes in 5q- cells is relevant to the abnormalities in hematopoiesis. We have shown that expression of the GM-CSF gene is markedly reduced in monocytes of patients with the 5q- refractory anemia syndrome although expression of M-CSF, G-CSF and the c-fms genes appears to be normal (Individual project: "Physiological role of the hematopoietic growth factor, GM-CSF"). To more specifically investigate the role of GM-CSF in normal hematopoiesis, we are developing vectors capable of expressing sequences complementary to GM-CSF mRNA (anti-sense RNA) on transfer into target cell populations.

The strategy of retroviral mediated gene transfer provides another avenue to explore the mechanisms of action of hematopoietic growth factors in normal hematopoiesis and of their mutated forms in hematopoietic malignancies. We have utilized a retroviral vector containing the murine IL-3 gene to transfer and express this gene in the hematopoietic stem, progenitor and precursor cells of mice (Individual project: "Retroviral transfer of the interleukin 3 gene causes a myeloproliferative syndrome"). Such mice develop a severe myeloproliferate syndrome with hematological features reminiscent of human chronic myelogenous leukemia. Detailed investigation of the pathogenesis has shown that the syndrome can arise by an autocrine or paracrine mechanism. Stem cell transfer and expression has been documented by serial passage into secondary recipients. A new generation of retroviral vectors is being developed in which the IL-3 gene and a known mutated proto-oncogene are incorporated. Our interest is defining the minimal requirements needed for development of acute leukemia and the role of growth factor genes and autocrine mechanisms in leukemogenesis. The work has been extended to examine the interleukin-6 gene as its product has been shown to have major roles in lymphocyte biology, antibody production and very early hematopoiesis (Individual project: "Role of the IL6 gene in hematopoiesis").

Our data has indicated that the autocrine mechanism of IL-3 in factor independent cells involves internal binding to the receptor. Using in vitro mutagenesis, we are creating modified genes that encode various forms of the growth factor in an effort to disassociate secretion from internal receptor interaction. These experiments are designed to more fully test the hypothesis regarding the role of internal ligand receptor interaction in the autocrine mechanism.



Furthermore, acquisition of growth factor independence and autocrine growth without secretion of the growth factor would be a major advantage in our in vivo studies of leukemogenesis. Separation of the effects of internally produced growth factor from the paracrine and endocrine effects of secreted growth factor would make these experiments much more interpretable.

Nucleic acid sequences complementary to messenger RNA (anti-sense sequences) may inhibit gene expression by blocking RNA processing or translation. Various model systems have been developed that reproducibly show the effect of c-fos or c-myc anti-sense sequences on cell proliferation and/or differentiation (Individual project: "Use of anti-sense RNA or DNA to inhibit gene expression"). We are attempting to extend these results by inserting coding sequences for anti-sense mRNA into retroviral vectors. We have also initiated a major effort to utilize anti-sense RNA to inhibit replication of the HIV virus (Individual project: "Inhibition of HIV replication in T-lymphocytes by anti-sense RNA sequences"). High titer retroviral stocks capable of transferring the anti-sense transcriptional unit into T-lymphocytes have been developed and we have shown high level expression of anti-sense RNA in polyclonal lymphoid populations. Nonetheless, these cells retain their sensitivity to HIV infection. Other portions of the HIV genome are being tested and new vector designs are being formulated in an effort to maximize anti-sense RNA production. Further, we anticipate using certain sequences from the viral genome that bind trans-acting factors encoded by HIV in an effort to competitively inhibit the function of these factors during productive infection. Although these experiments are labor intensive and not insured of success, the importance of the problem of acquired immunodeficiency syndrome has prompted us to continue this effort.

The dihydrofolate reductase gene is constitutively expressed in all cells and the level of expression is modulated during the cell-cycle (Individual project: "Characterization of the gene for human dihydrofolate reductase"). The promoter of the gene is found in a methylation free island in chromatin. Recent data in the laboratory has established that there is a second gene the promoter of which is within this methylation free island. The transcript is transcribed from the DNA strand opposite from that of the DHFR gene. The transcriptional start sites of the two genes are within 110 base pairs of one another and their promoters appear to share common sequence elements. The cDNA for the gene on the other strand from DHFR has been molecularly cloned and sequenced. It encodes for a large, slightly basic protein the identity of which is unclear after comparison to the data in the DNA and protein sequence banks.

A major focus of interest in the laboratory is the pathogenesis and treatment of aplastic anemia. Previous studies have implicated activated T-lymphocytes in the pathogenesis of aplastic anemia with gamma interferon the mediator of a suppressive effect on hematopoiesis. Recent experiments have shown that G-CSF could overcome gamma interferon's negative effect on myelopoiesis and IL-3 its effect on erythropoiesis (Individual project: "Lymphocytes and





lymphokines in aplastic anemia"). Monocytes from patients with aplastic anemia have been shown to be defective in their production of interleukin-1. This may be relevant to pathogenesis as IL-1 is known to stimulate release of hematopoietic growth factors by bone marrow stromal cells. During the past year a clinical protocol utilizing cyclosporine alone or in combination with prednisone for the treatment of refractory aplastic anemia has been completed. Thirty-five percent of the patients exhibited a hematologic response despite their previous lack of response to anti-thymocyte globulin. A clinical trial of the use of GM-CSF in the treatment of aplastic anemia has also been initiated.

The B19 parvovirus, discovered only 10 years ago, has been shown to be the cause of transient aplastic crisis of chronic hemolytic disease and fifth disease, a common childhood exanthem. Our work has focused on both the molecular biology and clinical spectrum of this virus (Individual project: "B19 (human) parvovirus"). A major advance has been the development of a cell line that synthesizes the viral capsid proteins. The sequences that encode these proteins were incorporated into an expression vector that included the human dihydrofolate reductase gene. Amplification of the introduced DNA in Chinese hamster ovary cells resulted in the high level of production of capsid proteins. Electron microscopy documented formation of viral capsids. This cell line provides a source of protein for use in structural studies and potentially, for the development of an effective vaccine.

The clinical spectrum of B19 induced disease is not yet fully defined. Recognized in the past year is the frequency with which this virus can cause chronic bone marrow suppression, primarily of erythropoiesis. This has been seen in patients with HIV infection, acute leukemia following treatment, congenital defects in antibody synthesis, and in patients without overt compromise of the immune system.

The Epstein-Barr virus (EBV) has been associated clinically with some cases of aplastic anemia (Individual project: "Viruses in aplastic anemia"). Molecular and immunological evidence of EBV infection has been documented in six patients with aplastic anemia and two patients with red cell aplasia. The pattern of restriction fragments obtained on Southern analysis of DNA from marrow cultures of aplastic anemia patients has been more similar to that of a non-transforming strain of EBV than to the typical transforming EBV of infectious mononucleosis. EBV appears to infect erythroid progenitors (BFU-E) making such cells susceptible to suppression by EBV sensitized lymphocytes.

A major collaborative study has been initiated to define the chromosomal location of the locus, mutant alleles of which can lead to hypertrophic cardiomyopathy (Individual project: "Mapping of the hypertrophic cardiomyopathy locus"). In many families, this clinical condition segregates as a dominantly inherited trait. We have assembled clinical information and DNA from five large families. Unique probes that detect highly polymorphic markers obtained through



collaborative interactions with Ray White of the Howard Hughes Institute Laboratory in Salt Lake City, will be used to identify the position of the HCM locus. The study involves a collaborative effort on the part of the Cardiology and Hematology Branches in the NHLBI and the genetic expertise provided by the Clinical Epidemiology Branch of the NCI.

In summary, the work of the Branch has expanded greatly and refocused on several problems of immense importance. Genetic therapy for hemoglobin disorders has become a realistic goal and is being vigorously pursued. Our knowledge of anti-sense inhibition of gene expression has suggested that this might be a useful treatment of AIDS and we have expanded our effort to determine whether this is true. Hematopoiesis has become amenable to the tools of molecular biology as the genes for hematopoietic regulators are defined and characterized. Experimental models of leukemogenesis are now feasible using defined growth factor genes and those with oncogenic potential. Many new avenues of investigation have been opened and our energies have been re-directed to take advantage of these opportunities to understand and treat human disease.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02208-14 CHB

## PERIOD COVERED

October 1, 1987-September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Iron Chelation and Transfusional Hemachromatosis

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Arthur W. Nienhuis, Chief, Clinical Hematology, CHB, NHLBI

Others: Patricia Griffith, Clinical Nurse Specialist, CHB, NHLBI

Janice Kimball, Clinical Nurse Specialist, CHB, NHLBI

W.F. Anderson, M.D., Branch Chief, LMH, NHLBI

Gary Brittenham, M.D., Division of Hematology, Cleve. Gen Hosp.

H. Strawczynski, M.D., Dir., Chronic Care Clinic, Montreal

Children's Hospital, Montreal, Quebec, Canada

Evan Tucker, M.D., Senior Investigator, CB, NHLBI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Clinical Hematology

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS

0.5

## PROFESSIONAL

0.5

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither☒ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

These studies are designed to evaluate the clinical benefits achieved by iron chelation in patients with chronic iron overload. Desferoxamine is administered by subcutaneous infusion and iron removal is determined by measurement of the serum ferritin and periodic non-invasive measurement of liver-iron concentration. Clinical status is evaluated by standard parameters including non-invasive testing of cardiac and endocrine function as indicated by the patients age and risk category. The study is designed to document the natural history of severe beta thalassemia, treated effectively with regular transfusions and chelation therapy tailored to the patient's clinical status. Fifty patients, followed for 5 or more years, have now been characterized with respect to chelation compliance, cardiac disease, endocrine dysfunction and liver iron concentration. Dramatic improvement in cardiac function has been documented in three patients, treated with intensive intravenous chelation.

407



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02307-09 CHB

PERIOD COVERED

~~October 1, 1987 - September 30, 1988~~

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

~~USE OF VIRAL REGULATORY SEQUENCES TO FACILITATE GENE TRANSFER~~

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: David M. Bodine, Ph.D., Staff Fellow, CHB, NHLBI

Others: Arthur W. Nienhuis, Chief, Clinical Hematology Branch, NHLBI  
Thalia Papayannopoulou, Univ. of Washington, Seattle, WA  
George Stamatoyannopoulos, Div. Med. Genetics, Univ. of  
Washington, Seattle

COOPERATING UNITS (if any)

University of Washington, Seattle, Washington

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute

TOTAL MAN-YEARS:

2.0

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Retroviral mediated gene transfer is the most efficient means to introduce new genetic material into the eukaryotic genome, and as such is a powerful tool for studying gene expression as well as a model for gene replacement therapy. Previously we showed that a modified N2 virus will transfer the human globin gene to erythroid cell lines and to primary hematopoietic progenitors. Infected mouse bone marrow cells were transferred to suitable recipient animals and transient  $\beta$  globin expression was observed in some animals.

We have analyzed 131 primitive progenitor colonies (CFU-S), 58 of which contained an intact retrovirus, 56 of these (96%) expressed human  $\beta$  globin mRNA at approximately 1% the level of mouse  $\beta$  globin mRNA. Our previous conditions seemed to infect only progenitor cells rather than pluripotent stem cells. Therefore we have modified our infection procedure to include pretreatment of the donor animals with 5FU, and infection of the marrow in the presence of growth factors thought to promote stem cell cycling. Using the growth factor IL-3 alone approximately 1/3 of the CFU-S (primitive progenitors) were infected and 3/31 mice were synthesizing human  $\beta$  globin mRNA and protein 4 months post transplantation. Using a combination of IL-3 and IL-6, 77% of the CFU-S were infected and 8/21 mice were synthesizing mRNA and protein 4 months post-transplantation.

410





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02310-08 CHB

## PERIOD COVERED

October 1, 1987-September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Characterization of the Gene for Human Dihydrofolate Reductase

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Takashi Shimada, M.D., Ph.D., Visiting Associate, CHB, NHLBI

Others: Hiroyuki Fujii, M.D., Ph.D., Visiting Fellow, CHB, NHLBI

Arthur W. Nienhuis, M.D., Branch Chief, CHB, NHLBI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Clinical Hematology Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

8.0

## PROFESSIONAL:

2.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

An RNA protection assay using strand specific probes identified RNA molecules that were initiated at position -92 relative to the Cap site of the human dihydrofolate reductase major transcript but transcribed from the opposite DNA strand. The upstream gene transcript was about 5 kilobase long, polyadenylated and detectable in HeLa, K562 and HL60 cells. A cDNA clone of the upstream gene was isolated. Sequence data suggested that the gene encodes a protein with a molecular weight of 11 kilodalton. A transient assay using the CAT gene as a reporter showed only 165 base pairs are sufficient for activity of either of the two opposite strand promoters.

Using a new high efficient DHFR minigene, we have developed a mammalian expression system by which the co-introduced gene is amplified in a short period and is expressed at a very high level. We have already succeeded in creating CHO cell clones which overproduce the human parvovirus capsid proteins and the human immunodeficiency virus envelope protein.

4/3



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02313-06 CHB

PERIOD COVERED

~~October 1, 1987 - September 30, 1988~~

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

~~Functional Mapping of Globin Gene Promoters~~

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Henry J. Lin, M.D., Medical Staff Fellow, CHB, NHLBI

Others: Kevin T. McDonagh, M.D., Medical Staff Fellow, CHB, NHLBI

Paul Ney, M.D., Medical Staff Fellow, CHB, NHLBI

Austine Moulton, Research Assistant, CHB, NHLBI

Amanda Cline, Research Assistant, CHB, NHLBI

Arthur Nienhuis, M.D., Branch Chief, CHB, NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER:

3.0

0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Our aim is to understand mechanisms involved in globin gene control, particularly developmental switching. The human K562 cell line mimics the embryonic and fetal erythroid cell milieu and is thus useful in studying molecular mechanisms controlling the human fetal  $\gamma$ -globin genes. Earlier we showed that a  $\gamma$ -globin gene promoter fragment spanning positions -259 and -137 activated the nonfunctional  $\beta$ -globin promoter in these cells. We therefore constructed a series of composite promoters, each containing a fragment from within this 120 base pair region joined to a  $\beta$ -globin promoter, and assessed their function in K562 cells. The assays previously used, however, proved to be inadequate for fine functional mapping. Modifications are currently being developed.

418



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02314-06 CHB

## PERIOD COVERED

October 1, 1987 - September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Function of proto-oncogenes in Human Hematopoietic Cells

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Elise A. Feingold, Ph.D., Guest Worker, CHB, NHLBI

Others: Arthur W. Nienhuis, M.D., Chief, CHB, NHLBI

Angel W. Lee, M.D., Ph.D., Medical Staff Fellow, CHB, NHLBI

Philip J. Browning, M.D., Medical Staff Fellow, NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Clinical Hematology Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The receptor for the monocyte-macrophage specific growth factor, CSF-1 (c-fms) and its viral homologue, v-fms, are transmembrane glycoproteins that exhibit tyrosine kinase activity. We have been attempting to identify the significant differences between v-fms and c-fms that contribute to the transforming properties of the viral gene. We previously reported studies on a number of v-fms/ human c-fms hybrid genes in which we found that the c-fms carboxyl terminal sequences appear to act in the negative regulation of the receptor. To more fully study the differences between v-fms and c-fms, we have cloned a full length mouse c-fms cDNA and are introducing specific amino acid substitutions with the aim of examining the effect of these changes in vivo. We have also constructed a chimeric receptor between the human epidermal growth factor (EGF) receptor ligand binding and transmembrane domains and the human c-fms cytoplasmic region including the tyrosine kinase domain and the carboxyl terminus. Since hematopoietic cells do not express the receptor for EGF, the chimeric receptor can be introduced into these cells and the cellular response to EGF can be examined. In addition, it may be possible to provide an inducible growth advantage to cells that express this chimera. To date, this gene has been introduced into both NIH 3T3 and IL-3 dependant 32D cells. It has been found to be expressed on the cell surface and to be of the expected size. Further, this receptor exhibits autophosphorylation that can be further stimulated by the addition of EGF. The cells bearing the chimeric receptor, however, do not exhibit a mitogenic response upon addition of EGF. The reason for this block is unknown and will be the subject of further investigation.

421



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02315-06 CHB

## PERIOD COVERED

October 1, 1987-September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Lymphocytes and lymphokines in aplastic anemia

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Neal S. Young, M.D. Section Chief, CHB, NHLBI

Others: Shinji Nakao, M.D., Visiting Fellow, CHB, NHLBI

Stacie Anderson, Medical Technologist, CHB, NHLBI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Clinical Hematology

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2.5

## PROFESSIONAL:

2.5

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☒ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Clinical studies have focused the development of more specific and standard therapy for patients with aplastic anemia. A study of cyclosporine has demonstrated that patients with refractory aplastic anemia can improve hematologically with this form of T-cell directed immunosuppression. Cyclosporin A resulted in approximately 35% hematologic response rate. The addition of prednisone and the maintenance of adequate blood levels of cyclosporine appeared to be important for success of this regimen. Studies of hematopoietic growth factors in aplastic anemia have begun with the treatment of three patients with severe disease, including severe neutropenia and frequent bacterial infections using recombinant human GM-CSF. In laboratory studies, monocytes from patients with aplastic anemia have been shown to be very poor producers of interleukin 1 (Il-1), a principle regulator of immune and possibly also of hematopoietic cell proliferation. Il-1 activity in monocyte cultures was correlated to the degree of neutropenia and improved with successful therapy of aplastic anemia. The ability of hematopoietic growth factors to overcome the negative effects on hematopoietic cell proliferation of gamma interferon have been investigated. G-CSF had no effect on gamma interferon suppression of colony formation. GM-CSF could overcome gamma interferon's negative effect on myelopoiesis, and Il-3 its effect on erythropoiesis, consistent with the relative activity of these factors on CFU-GM and BFU-E<sup>-</sup> derived colony formation.

426





## NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

701 HL 02319-05 CHB

PERIOD COVERED  
October 1, 1987-September 30, 1988TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
B19 (Human) Parvovirus

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Neal Young, M.D.

Others: G. Kurtzman, M.D., Senior Staff Fellow, CHB, NHLBI

S. Kachigaya, M.D., Visiting Fellow, CHB, NHLBI

J. Ayub, M.D., Chemist, CHB, NHLBI

T. Shimada, M.D., Visiting Associate, CHB, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH  
Clinical Hematology

SECTION

INSTITUTE AND LOCATION  
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

3.4

PROFESSIONAL:

2.4

OTHER:

1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☒ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Studies of the B19 parvovirus can be separated into clinical and basic laboratory work. In the laboratory, interesting molecular features of the B19 parvovirus have been elucidated using in vitro transcription and translation systems. We have identified the precise relationship between specific virus transcripts and protein products and demonstrated that multiple upstream AUG triplets help to regulate the quantities of viral capsid protein produced. A major problem in B19 parvovirus studies has been the lack of a simple, productive culture system for production of antigen. This difficulty has been overcome by creation of a viral capsid-producing cell line. Chinese hamster ovary cells have been transfected with DNA from the right side of the B19 genome. These cells produce B19 parvovirus empty capsids as well as large quantities of the major and minor capsid species. Clinical studies have demonstrated that B19 parvovirus replicates in cells of the peripheral blood during acute infection. B19 persistence has been shown in at least three patient populations; patients with congenital immunodeficiency, children with acute lymphocytic leukemia in remission on chemotherapy, and patients with acquired immunodeficiency syndrome. A common immunologic finding in patients with persistent disease is the absence of neutralizing antibody, which is paralleled by a low or absent binding of serum to virus capsid proteins on Western immunoblot analysis. Patients with persistent B19 parvovirus infection usually manifest their illness only by chronic red cell aplasia. The diagnosis may be established clinically based on characteristic bone marrow morphology and then confirmed by appropriate DNA studies. One patient with at least a three and probably as long as 12 year course of chronic parvovirus infection has been "cured" by the infusion of commercial immunoglobulin containing antibodies to parvovirus.

430



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 HL 02320-05 CHB
PERIOD COVERED October 1, 1987-September 30, 1988		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Pharmacologic Manipulation of HbF Synthesis		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)  PI: Kevin T. McDonagh, M.D., Medical Staff Fellow, CHB, NHLBI Others: Arthur W. Nienhuis, M.D., Branch Chief, CHB, NHLBI Brian Agricola, Animal Technician, Section on Animal Surgery, CHB, NHLBI Ellen Byrne, Animal Technician, CHB, NHLBI Robert Hoyt DVM, Chief, Section on Animal Surgery, CHB, NHLBI		
COOPERATING UNITS (if any) Laboratory of Chemical Biology, NIDDK Griffin Rodgers, M.D., Constance T. Noguchi, Ph.D, and Alan Schechter, M.D.		
LAB/BRANCH Clinical Hematology Branch		
SECTION		
INSTITUTE AND LOCATION National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS: 1.0	PROFESSIONAL: 0.5	OTHER: 0.5
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither. <input checked="" type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <div style="text-align: justify;"> <p>These studies are designed to determine the ability of various substances to induce HbF synthesis in experimental animals and humans. Because of its safety and demonstrated activity, we have initiated an expanded phase I-II study of hydroxyurea. The purpose of this study is to further define the frequency and magnitude of the response of individual patients and to identify significant toxicity.</p> <p>During the past several years various hematopoietic growth factors have been molecularly cloned and large quantities of purified, biologically active material are available for clinical testing. Those growth factors with demonstrated activity on erythroid precursors, such as erythropoietin, IL3 and GM-CSF, have the potential for stimulating HbF synthesis. Our studies in Rhesus monkeys have shown that erythropoietin increases HbF production modestly when administered alone, but may be augmented by a preceeding course of hydroxyurea, IL3 or GM-CSF.</p> </div>		



## NOTICE OF INTRAMURAL RESEARCH PROJECT

701 HL 02321-04 CHB

## PERIOD COVERED

October 1, 1987-September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Use of Antisense RNA or DNA to Inhibit Gene Expression

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:Robert L. Redner, M.D., Medical Staff Fellow, CHB, NHLBI

Others:Gail Osawa, Chemist, CHB, NHLBI

Stacie Anderson, Medical Technologist, CHB, NHLBI

Arthur W. Nienhuis, M.D., Branch Chief, CHB, NHLBI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Clinical Hematology Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, Md. 20892

## TOTAL MAN-YEARS:

4.0

## PROFESSIONAL:

3.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

These studies are designed to analyze the effects of oncogene expression on cell growth and differentiation. We have previously shown that inducible antisense c-fos constructs can inhibit fibroblast growth, and that single stranded antisense oligomers can induce differentiation of HL-60 cells. Antisense c-myc constructs only mildly inhibit fibroblast growth, possibly because of an inability to obtain a suitable ratio of antisense:sense RNA. A more useful model system is the 32-D cell line, in which removal of interleukin-3 from the media induces a fall in c-myc and growth arrest; conversely, addition back of Il-3 induces a rise in c-myc with a subsequent re-entry into the cell cycle. Induction of antisense c-myc RNA during Il-3 induced quiescence should allow more detailed study of the role of c-myc in the cell cycle.

439



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02324-04 CHB

## PERIOD COVERED

October 1, 1987-September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Identification of Cis

and Trans-acting Elements that Regulate Human Gamma Gene Expression

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: David M. Bodine, Ph.D., Staff Fellow, CHB, NHLBI

Others: Arthur W. Nienhuis, Chief, Clinical Hematology Branch, NHLBI

Peter C. Hoppe, Ph.D., Senior Staff Scientist, The Jackson  
Laboratory, Bar Harbor, Maine

## COOPERATING UNITS (if any)

## LAB/BRANCH

Clinical Hematology Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2.0

## PROFESSIONAL:

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have been investigating the regulation of the human fetal ( $\gamma$ ) and adult ( $\beta$ ) globin genes to identify the transcriptional control signals for these genes.

Using transgenic mice we have shown that the transcription of closely linked human  $\gamma$  and  $\beta$  globin genes is developmentally regulated, demonstrating that the developmental regulatory sequences are located close to each gene. Recently we have shown that the human  $\beta$  globin gene acquires DNase I hypersensitive sites that are concordant with those of the previously uncharacterized mouse adult  $\beta$  globin genes. In contrast the  $\gamma$  gene remains relatively hypersensitive throughout development, and independent of the presence of hypersensitive sites in its two evolutionary homologues in the mouse,  $\beta^{h0}$  and  $\beta^{h1}$ . We are attempting to corroborate our analysis of globin promoter function in K562 cells by demonstrating correctly initiated globin CAT mRNA in transfected cells. We have developed a new CAT gene that contains the 3' RNA processing signals and a set of RNase protection assay probes to achieve this goal.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

701 HL 02328-03 CHB

PERIOD COVERED October 1, 1987 - September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
Modification of Retroviral Targeting via Hybrid Envelope Proteins

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Timothy M. Browder, M.D., Senior Staff Fellow, CHB  
Takashi Shimada, M.D., Ph. D., Visiting Associate, CHB  
John Abrams, Ph.D., DNAX, Palo Alto, CA  
Arthur W. Nienhuis, M.D., Branch Chief, CHB, NHLBI

COOPERATING INSTITUTIONS (if any)  
DNAX Research Institute of Molecular and Cellular Biology, Inc.  
901 California Avenue, Palo Alto, CA 94304-1104

LAB/BRANCH Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS: 2.0

PROFESSIONAL: 0.2

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

These experiments are designed to test whether structural alterations of retroviral envelope glycoproteins can direct virus targeting for tissue-specific gene transfer. One avenue explores the expression of a hybrid envelope gene which encodes for a murine pluripotent colony stimulating factor, interleukin 3 (IL3). Such a virus could then specifically target bone marrow cells expressing the high affinity IL3 receptor (IL3R). A second avenue explores construction of a packaging line specific for CD4-positive lymphocytes to introduce HIV anti-sense vectors. Both packaging lines should then allow tissue-specific viral infection of therapeutic genes in vivo without the attendant risks of marrow transplantation.

444



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02330-02 CHB

## PERIOD COVERED

October 1, 1987-September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mapping of Hypertrophic Cardiomyopathy Locus

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Arthur W. Niennhuis, M.D., Chief, Clinical Hematology Branch, NHLBI

Others: Neal D. Epstein, M.D., Clinical Hematology Branch, NHLBI

Stephan E. Epstein, M.D., Cardiology Branch, NHLBI

Henry J. Lin, M.D., Clinical Hematology Branch, NHLBI

Barry J. Maron, M.D., Cardiology Branch, NHLBI

John J. Mulvihill, M.D., Clinical Epidemiology Branch, NCI

Dilys M. Parry, Ph.D, Clinical Epidemiology Branch, NCI

Ray White, Ph.D, Howard Hughs Med.Inst., Univ. of Utah, S.L.C., Utah

## COOPERATING UNITS (if any)

Howard Hughs Medical Institute, Univ. of Utah School of Medicine,  
Salt Lake City, Utah; Cardiology Branch, NHLBI; Clinical Epidemiology  
Branch, NCI

## LAB/BRANCH

Clinical Hematology Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, Md. 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☒ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The purpose of this protocol is to determine the chromosomal locus or loci of the genes responsible for hypertrophic cardiomyopathy (HCM). This heart disease is of obscure etiology and may range in its presentation from an incidental finding to sudden death. The gold standard in the diagnosis of HCM remains echocardiography. Approximately one-half of the cases are sporadic and one-half are familial. Previous family studies have suggested an autosomal dominant pattern of inheritance in familial cases. We have ascertained five large kindreds affected with the disease. An autosomal dominant pattern of inheritance is evident in all pedigrees. We have studied all five families with echocardiography and have collected blood for DNA extraction and blood protein polymorphisms determinations. This allows us to use the technique of genetic linkage analysis to map the gene. This technique has been already used by others to map the genes responsible for diseases such as cystic fibrosis, Huntington's Chorea, Alzheimers disease and bipolar affective disorder. Once the gene has been localized questions of genetic heterogeneity can be addressed by studying patients outside of these two families. Ultimately the marker which establishes the chromosomal location can be used to obtain the gene itself.

447



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02331-02 CHB

## PERIOD COVERED

October 1, 1987-September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Inhibition of HIV Replication in T-Lymphocytes by Anti-sense RNA Sequences

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Takashi Shimada, M.D., CHB, NHLBI

Others: Hiroyuki Fujii, M.D., CHB, NHLBI

Bernhard Maier, Ph.D., CHB, NHLBI

Timothy Browder, M.D., CHB, NHLBI

Arthur W. Nienhuis, M.D., CHB, NHLBI

Seiji Hayashi, M.D., COP, DCT, NCI

Hiroaki Mitsuya, M.D., COP, DCT, NCI

Samuel Broder, M.D., COP, DCT, NCI

## COOPERATING UNITS (if any)

Clinical Oncology Program, Division of Cancer Treatment, National Cancer Institute

## LAB/BRANCH

Clinical Hematology Branch/Molecular Biology Section

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS: 1.0

PROFESSIONAL 1.0

OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☒ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Acquired immunodeficiency syndrome is caused by a human immunodeficiency virus (HIV) that infects and destroys helper T-lymphocytes. These experiments are designed to test whether RNA sequences complimentary to portions of the HIV genome (anti-sense RNA) can block HIV replication or gene expression thereby creating T-lymphocytes that are resistant to the cytopathic affects of HIV. We have established a new construction scheme of expression vectors and tested various promoters and vectors systematically. The human B19 parvovirus promoter was shown to be the most potent promoter so far tested in human T-cell lines. Using the high titer retrovirus vectors containing the B19 promoter driven anti-sense transcriptional units, we have succeeded in creating T-cell lines expressing certain HIV anti-sense sequences at high levels. Several clones have been already tested for their ability to withstand infection by the HIV. However; There was no significant difference in the rate of viral replication between these clones and mock-infected T-cells. We are currently tested whether anti-sense sequences of other portions of the HIV genome can inhibit virus replication. We have also designed several new retrovirus vectors to increase the level of expression of anti-sense sequences. If an effective anti-sense expression retrovirus vector is identified in culture system, the vector may be tested in primates.

450



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02332-02 CHB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Viruses and Aplastic Anemia

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Neal S. Young, M.D., Chief, Cell Biology Section, CHB, NHLBI

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## COOPERATING UNITS (if any)

## LAB/BRANCH

Clinical Hematology Branch

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1.35

## PROFESSIONAL:

1.25

## OTHER:

.1

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☒ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Because Epstein-Barr virus (EBV) has been associated clinically with some cases of aplastic anemia, we have sought the presence of this virus using immunologic and molecular methods in the bone marrows of patients with pancytopenia and pure red blood cell aplasia. In 6 patients with aplastic anemia and 2 patients with red cell aplasia (one with prolonged aplastic crisis of sickle cell disease, the second an atypical relapse in Diamond-Blackfan syndrome), EBV has been demonstrated by the presence of nuclear antigen (EBNA) with immunofluorescence and of DNA by in situ hybridization and Southern analysis. The pattern of restriction fragments obtained on Southern analysis of DNA from marrow cultures of aplastic anemia patients has been more similar to a non-transforming type of EBV than to the typical transforming EBV of infectious mononucleosis. EBV appears to be capable of infecting an hematopoietic progenitor cell at the BFU-E stage. When normal bone marrow is exposed to EBV or autologous EBV lymphoblastoid cell lines, virus can be demonstrated in the isolated progeny of erythroid colonies by immunofluorescent and molecular methods. EBV is not directly cytotoxic to progenitor cells. However, T lymphocytes that have been exposed to EBV are capable of specifically inhibiting erythroid colony formation by EBV infected bone marrow. These results suggest that hematopoietic suppression associated with EBV may be the result of an immune response with activation of suppressor T lymphocytes. Parallel studies with cytomegalovirus have suggested that CMV is not capable of inhibiting or infecting hematopoietic progenitor cells.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02333-01 CHB

## PERIOD COVERED

October 1, 1987-September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structure and Function of the Murine M-CSF Receptor

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Angel W. Lee, M.D., Ph.D., Medical Staff Fellow, CHB, NHLBI

Others: Arthur W. Nienhuis, M.D., Branch Chief, CHB, NHLBI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Clinical Hematology

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS

1.0

## PROFESSIONAL:

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The mechanism of growth factor and receptor interaction is the objective of this project. We utilize the murine CSF-1 receptor as a prototypic growth factor receptor. As the initial step, the receptor has been cloned, sequenced and subsequently introduced into tissue culture cells via retroviral gene transfer techniques to demonstrate its functionality. The specific question being addressed is whether ligand (growth factor) activation of the receptor as an enzyme (tyrosine kinase) occurs via an intramolecular or intermolecular mechanism. In one case we modify the receptor (at the cDNA level) such that the extracellular ligand binding domain is structurally separated from the rest of the receptor although still covalently attached. In this manner we hope to disrupt the transmission of any intramolecular conformational signal. In the second case, we construct a hybrid glycoporphin A - CSF-1 receptor that can be aggregated by an antiglycophorin antibody in order to study enzyme activation via an intermolecular process.

We are also in the process of raising polyclonal antibodies to synthetic peptides representing different parts of the receptor for use as conformational probes.

Finally, to enable biochemical and biophysical studies to be performed on growth factor receptors, we are overexpressing the extracellular domain of the CSF-1 receptor in mammalian and insect cell systems.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02334-01 CHB

## PERIOD COVERED

October 1, 1987-September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

DNA Binding Proteins That Interact with Gamma Globin Gene Promoters

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Kevin T. McDonagh, M.D., Medical Staff Fellow, CHB, NHLBI

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## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The genes for gamma globin are examples of genes with strict tissue specific and developmentally specific patterns of expression. Our laboratory has found that a fragment of the promoter region of the gamma globin gene is critical for appropriate regulation of expression. Using extracts of nuclear proteins from the human erythroleukemia K562 cell line, we have found that there are several regions within the promoter which interact with potential transcriptional regulatory proteins. The proteins which bind to these regions are being purified and mutant promoters which contain ablated protein binding sites are being evaluated in a variety of transcriptional assays.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02335-01 CHB

## PERIOD COVERED

October 1, 1987 - September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) ~~Retroviral Transfer of the~~  
Interleukin-3 Gene Causes a Myeloproliferative Syndrome

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Cynthia Dunbar M.D. Medical Staff Fellow  
Others: Peter Wong Ph.D. Guest Worker  
David Bodine Ph.D. Senior Staff Fellow  
Timothy Browder M.D. Senior Staff Fellow  
Arthur Nienhuis M.D. Branch Chief

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## LAB/BRANCH

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TOTAL MAN-YEARS

1.0

PROFESSIONAL:

OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Dysregulated expression of hematopoietic growth factor genes may produce uncontrolled proliferation and abnormal differentiation of hematopoietic progenitors, and potentially be a factor in the production of leukemias. Retroviral vectors offer a highly efficient method for the introduction of growth factor genes, freed of normal regulatory constraints, into hematopoietic cells. Interleukin-3 is known to support the growth of early hematopoietic progenitors from multiple lineages in vitro. We had previously shown that a retroviral vector carrying the IL3 gene (N2-IL3) could infect myeloid cell lines and hematopoietic progenitors, rendering them factor-independent in vitro. When lethally irradiated or congenitally anemic mice were transplanted with N2-IL3-infected bone marrow or fetal liver cells, a striking myeloproliferative syndrome resembling human chronic myelogenous leukemia developed. The syndrome was characterized by a marked elevation of the white blood cell count, bone marrow myeloid hyperplasia with normal maturation, and massive infiltration and enlargement of the spleen and liver. Molecular analysis showed that there was proliferation of and repopulation by one or several infected stem cell clones. Bone marrow from these mice repopulated secondary recipients, and recreated in them the identical syndrome with the same stem cell clone or clones contributing. One unusual primary animal developed the syndrome by a paracrine or endocrine mechanism, with stimulation of the recipient's endogenous marrow as well as proliferation of the infected stem cell clones. Further studies are in progress to assess the contribution of autocrine versus paracrine or endocrine mechanisms in this syndrome, and to investigate whether the concurrent introduction of a transforming oncogene will produce an acute instead of a chronic leukemia.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02336-01 CHB

## PERIOD COVERED

October 1, 1987 - September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Physiological Role of the Hematopoietic Growth Factor GM-CSF

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I: Debra A. Cockayne, Ph.D., Staff Fellow, CHB, NHLBI

Others: Arthur W. Nienhuis, M.D., Chief, CHB, NHLBI

Stacy Anderson, Medical Technologist

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Clinical Hematology Branch

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## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects☒ (b) Human tissues☐ (c) Neither☒ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

5q- syndrome is a transfusion dependent refractory anemia associated with abnormal megakaryocytopoiesis and an acquired partial deletion of the long arm of chromosome 5, that manifests itself in the myeloid lineage of hematopoietic cells. Several growth factor and growth factor receptor genes have been localized to this region of chromosome 5, including the genes for IL-3, GM-CSF, M-CSF and c-fms. Several mechanisms have been proposed to address the relationship between the loss of one allele of several hematopoietic regulatory genes and the abnormal hematopoietic differentiation observed in patients with the 5q- syndrome. We have been attempting to discriminate between two possible etiological mechanisms, one being that of a gene dosage effect and the other being that the loss of a wild type allele has unmasked a receive mutant allele in a specific gene. Our initial approach has been to ask whether a 5q- cell population can express the hematopoietic regulatory genes mapped to the 5q- deletion. This analysis is being applied to peripheral blood (PB) monocytes as these cells harbor the 5q- deletion and are known to express the genes for GM-CSF, M-CSF and c-fms during normal hematopoiesis. Steady state levels of monocyte RNA have been quantitated, before and after stimulation with lipopolysaccharide (LPS), by Northern blot analysis using gene specific cDNA probes. We have demonstrated that the expression of GM-CSF by 5q- monocytes differs markedly from that of normal monocytes. Following stimulation with LPS 5q- monocytes accumulate only 10% of the GM-CSF mRNA found in LPS stimulated control monocytes. No differences were observed however, in the level of expression of M-CSF, c-fms or G-CSF mRNA following LPS stimulation of either 5q- or normal control monocytes. We are presently carrying out in vitro transcription assays to further characterize the observed decrease in the steady state levels of GM-CSF mRNA that is produced by 5q- monocytes.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

701 HL 02337-01 CHB

## PERIOD COVERED

October 1, 1987-September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
Role of Interleukin-6 Gene in Hematopoiesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Stephen Brandt, Guest Worker, CHB, NHLBI

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LAB/BRANCH

Clinical Hematology

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Interleukin-6 (IL-6) is one of the principal mediators of the acute phase response and has important actions on early hematopoietic progenitors, hepatocytes, and on B- and T-lymphocytes. Excessive or inappropriate synthesis of IL-6 may be involved in the pathogenesis of rheumatoid arthritis and multiple myeloma. The objective of this work is to investigate the consequences of unregulated expression of IL-6 in normal lymphocytes, cell lines, and in intact animals. A series of retroviral vectors are being constructed that are capable of transferring the murine IL-6 coding sequence with the neomycin resistance gene, other growth factors with which IL-6 acts synergistically in vitro, and oncogenes implicated in the development of plasmacytomas. In addition, a modified retroviral vector with the murine immunoglobulin heavy chain enhancer replacing the Moloney viral enhancer element will be made with the objective of targeting the expression of these sequences to differentiating B-lymphocytes. These experiments should yield new information about the role of this cytokine in certain autoimmune and neoplastic disorders.

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ANNUAL REPORT OF THE  
HYPERTENSION-ENDOCRINE BRANCH  
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE  
October 1, 1987 through September 30, 1988

This year the Hypertension-Endocrine Branch has continued its basic and clinical research into some of the important aspects of the causes and therapy of hypertension. These studies have focused on a number of the neurohumoral and vasoactive systems that control the circulation.

I. Catecholamines and the sympathetic nervous system. Virtually every activity of our lives is associated with changes in the activity of the sympathoadrenomedullary system, changes which are continual, usually silent, and vital for preserving the constancy of the internal environment. The challenge for many years has not been the discovery of this system or its importance, especially in the elaboration of stress responses, but its role in the pathophysiology of disease. Our laboratory has developed several new techniques to determine the role of the sympathoadrenomedullary system in cardiovascular diseases. Thus we originally developed techniques for measuring plasma catecholamines, i.e., norepinephrine (NE) and epinephrine (E), making use of HPLC and electrochemical detection. However, we soon realized that inferences about sympathetic outflow can be erroneous when based on antecubital venous levels of NE, because the sympathetic innervation of the arm contributes importantly to NE in the venous drainage. Thus, we developed tracer pharmacokinetic procedures which have provided a more refined approach for estimating overall and regional sympathetic function. We have recently found that during the mental challenge involved in playing a video game that normal volunteers show a substantially increased total body NE spillover, with the increment related directly to the magnitude of the pressor and cardiac output responses. In contrast, antecubital venous NE was unchanged and unrelated to systemic hemodynamic responses. On the other hand, relatively young patients with essential hypertension had larger increments in total body NE spillover while playing the video game than did normotensive subjects, despite similar baseline total body NE spillover in the two groups. The magnitude of the pressor response for a given increment in total body NE spillover was normal in most patients, thus enhanced pressor responses during mental challenge in the hypertensives resulted mainly from increased sympathetically mediated NE release. The pressor responses in hypertensives were due to increased total peripheral resistance, whereas the pressor responses in normotensives were due to increased cardiac output. Since responses of arterial E were enhanced in the hypertensives, whereas responses of



corticotropin were not, both during mental challenge and during administration of the alpha receptor blocker, yohimbine, hypertensives did not appear to have exaggerated "stress responses" so much as increased sympathoadrenomedullary responsiveness. In fact, during yohimbine administration, about 1/3 of young hypertensives had marked pressor and sympathoadrenomedullary responses associated with various emotional experiences. These findings paralleled those in juvenile spontaneously hypertensive rats (SHR) in which the responses of plasma catecholamines to yohimbine were several fold larger than those of age-matched normotensive control rats. Thus the clinical and laboratory results supported the hypothesis of enhanced sympathoadrenomedullary responsiveness during the development of spontaneous hypertension.

By means of tracer pharmacokinetic procedures, we found that, while responses of total body NE spillover were enhanced in hypertensives during mental challenge, responses of forearm NE spillover were normal. The results, coupled with those of Esler and co-workers demonstrating increased cardiorenal NE spillover in young hypertensives, point to increased cardiorenal sympathetic responsiveness in essential hypertension. We have recently been able to make direct measurements of recorded renal nerve activity, renal blood flow, and arterial renal venous tritiated and endogenous NE in anesthetized, adrenal-demedullated rats during nitroprusside-induced hypotension, phenylephrine-induced hypertension, and blockade of ganglionic neurotransmission. We have obtained the first evidence that physiologic changes in directly recorded sympathoneural activity were related to changes in regional NE spillover. In humans, we have recently observed virtually identical proportionate, physiological increases in antecubital venous NE and muscle sympathoneural activity.

One possible cause of abnormal regional NE spillover is abnormal reuptake of NE via Uptake-I, the main mechanism for removal of endogenously released NE. By use of techniques developed previously, we found that regional removal of circulating NE by Uptake-I was unimportant in the forearm, but appeared to be the predominant means for removal of circulating NE in the heart. This may explain why fatal cardiac arrhythmias are so common in subjects with cocaine overdose, since cocaine is one of the most potent blockers of the Uptake-I mechanism.

Dihydroxyphenylglycol (DHPG) is the predominant intraneural metabolite of NE. We have obtained evidence demonstrating that plasma DHPG reflects the rate and site of NE metabolism in both man and laboratory animals. Combined measurement of endogenous and radiolabelled DHPG and NE during systemic intravenous infusion of



tritiated NE formed the basis for our first clinically applicable method of estimating the regional rate of reuptake of endogenously released NE. In conscious rats, we estimated the total body rate of neuronal reuptake of endogenous NE to be 1.45 nmol/kg/min. and in humans about 0.40 nmol/kg/min. In both species, this would represent about 10 times the total body rate of release of NE into the bloodstream. Thus, most of endogenously released NE is in fact removed by Uptake-I, with only a small proportion diffusing into the blood stream. We have found that in patients with hypertrophic cardiomyopathy (HCM), cardiac DHPG production was decreased for a given amount of regional NE spillover, indicating decreased neuronal reuptake of NE. This could be important in the pathophysiology of this disease because deficient Uptake-I activity would result in excessive or prolonged occupation of postsynaptic adrenal receptors during sympathetic stimulation.

We have made use of a fluorinated analog of NE, i.e., fluorodopamine, as a means of performing positron emission tomography (PET) scanning of the sympathetic nervous system. Our preliminary studies in animals show that this provides an in vivo assessment of sympathetic neuroanatomy and function. Thus after systemic intravenous administration of positron emitting fluorodopamine into dogs with unilateral superior cervical ganglionectomies, the pattern of positron emission indicated little, if any, uptake of fluorodopamine into the brain, consistent with an efficient blood brain barrier for catecholamines, while cranial positron emission was most intense in regions corresponding to the four salivary glands (known to have extensive sympathetic innervation). Much less positron emission was noted from the ganglionectomized side than from the intact side, demonstrating that positron emission was due to uptake of fluorodopamine into intact sympathetic nerve endings. The heart avidly removed and concentrated circulating fluorodopamine, providing striking PET imaging of the sympathetic innervation of the cardiac ventricles. Thus, PET imaging would appear to be clinically feasible at this time and may prove useful, not only in defining sympathetic neuroanatomy, but also in measuring regional NE turnover.

## II. DOPA, dopamine, and the dopaminergic nervous system.

Dihydroxyphenylalanine (DOPA) is the precursor of all endogenous catecholamines and the immediate product of the rate-limiting step in catecholamine biosynthesis. Plasma concentrations of DOPA are about 10 times those of NE. We have found evidence that plasma DOPA derives substantially from sympathetic nerve endings and the brain. Thus in normal volunteers we consistently noted arterial venous increments of plasma DOPA in the arm, indicating regional production of DOPA. While in sympathectomized limbs, no arterial venous increment in plasma DOPA was observed. We have noted a strongly positive relationship between the





arterial venous increments in DOPA and in DHPG across the brain in humans, indicating that release of DOPA into the blood stream by the brain was related to central neural turnover of NE. Similarly, the cardiac arteriovenous increment in plasma DOPA was directly related to the cardiac arteriovenous increment in plasma DHPG. Tyrosine hydroxylation (TH) is the rate-limiting step in catecholamine synthesis. We have performed a series of experiments in which we produced alterations in tyrosine hydroxylation to determine whether changes in plasma DOPA could reflect changes in catecholamine biosynthesis. These included manipulations causing hypotension, such as hemorrhage or administration of the direct-acting vasodilator, nitroprusside, which would be expected to increase TH activity due to markedly increased release of NE. We also gave prolonged infusions of a ganglionic blocker, which decreases NE formation and release, and is expected to decrease TH activity. Similarly, reserpinization, by depleting NE stores, increases TH activity, whereas acute administration of NE, by increasing cytoplasmic NE, decreases TH activity by feedback inhibition. In every case examined so far, where TH activity was expected to change, we found that plasma DOPA changed in the same direction.

We have extended our studies of DOPA and dopamine metabolism in both man and experimental animals. As reported previously, we have shown that when one increases the sodium intake of a patient from 9 mEq/day to 249 mEq/day, there is an increase in urinary dopamine and a concomitant increase in urinary DOPA. The interesting finding is that there is no change in plasma DOPA. These observations suggest that sodium administration increases the extraction of DOPA by the kidney without changing the delivery of DOPA to the kidney. It is presumed that this change in extraction of DOPA and the subsequent metabolism to dopamine is the basis for the increase in sodium excretion that we observe. Since dopamine excretion is abnormal in both salt-resistant (SR) and salt-sensitive (SS) hypertensive subjects, the possibility that abnormalities in DOPA metabolism were responsible was studied further. On a low sodium diet, DOPA excretion was twice normal in both SR and SS subjects, and dopamine excretion in SR was higher than normal. When sodium intake was increased, DOPA excretion increased in SR and SS by 47% and 96%, respectively (vs. 66% in normal subjects). However, urinary dopamine increased by only 18% and 15% respectively (vs. 49% in normal subjects). These results suggest that the conversion of DOPA to dopamine by the kidney is impaired in SS hypertensive subjects.

There appear to be special high affinity uptake sites for dopamine, especially in the brain. These recognition sites appear to constitute a molecular component of the dopamine uptake domain. We have found that one can modulate these binding sites for dopamine uptake by



receptor-receptor interaction and by post-translational modification of membrane proteins present in the synaptosomes. Thus glutamate was shown to potentiate the release of dopamine elicited by 50 mM potassium from striatal slices and this effect was attenuated by preincubating striatal slices with glutamate receptor blockers. One of the major sites of the dopaminergic nervous system is in the caudate nucleus, and destruction of the glutamatergic innervation in this portion of the brain led to an increase in sodium-dependent cocaine binding, while the affinity of radioligands for this binding site was similar in control and on the decorticated side. Thus the efficacy of these drugs was higher in slices prepared from decorticated brain hemispheres than from the contralateral control side. Thus we have shown a significant modulation of uptake of dopamine in this area and have also been able to coordinate this activity with activation of protein kinase C in these same neurons.

The role of GABA receptors in the function of adrenal chromaffin cells was studied in situ by autoperfusion of canine adrenal glands as a means of studying regulatory mechanisms for adrenal chromaffin cell secretion. We found that direct stimulation of GABA<sub>A</sub> receptors located on chromaffin cell membranes causes bursts of chloride channel opening that result in depolarization of chromaffin cell membranes and thereby facilitates the spontaneous release of neurotransmitters. It is proposed that this type of depolarization, involving bursts of chloride channel opening, may obtund the depolarizing effect of nicotinic receptor stimulation, one of the major stimuli for neurotransmitter release. Hence in the adrenal medulla, GABA can be viewed as a primary transmitter that modifies the effect of nicotinic receptor stimulation by acting on a different transducer system, presumably the chloride channel. These findings apply not only to catecholamine release, but also to release of met-enkephalin-like peptides. It will now be important to determine whether GABA also regulates the release of additional neuropeptides from chromaffin cells, including substance P and neuropeptide Y.

Digoxin is a potent stimulant for the release of catecholamines from chromaffin cells. Since digoxin is a potent inhibitor of Na<sup>+</sup>K<sup>+</sup>ATPase, we studied the role of this enzyme in catecholamine release from slices of bovine adrenal glands. We found only one homogeneous population of ouabain binding sites, which are located on the plasma membrane of chromaffin cells; this binding site was specific and could be displaced by other cardiac glycosides with an order of potency: ouabain > acetyldigoxin > digitoxin. Blockade of Na<sup>+</sup>K<sup>+</sup>ATPase with cardiac glycosides induced catecholamine release from isolated bovine chromaffin cells. This catecholamine release was both calcium- and temperature-dependent. The order of potency for cardiac glycosides was ouabain > strophanthidin > digitoxin. We next tried to correlate the



catecholamine release induced by cardiac glycosides to the  $\text{Na}^+\text{K}^+\text{ATPase}$  activity measured as  $^{86}\text{Rb}$  or  $^{42}\text{K}^+$  uptake in isolated chromaffin cells. The  $^{86}\text{Rb}$  uptake reached a plateau at 60 minutes. A similar course was obtained with  $^{42}\text{K}^+$ .  $^{86}\text{Rb}$  uptake was blocked in a dose-dependent manner by cardiac glycosides, with ouabain being the most potent drug. We found a discrepancy between the  $K_d$  obtained from binding studies and the  $\text{ED}_{50}$ 's that evoke catecholamine secretion. This suggests that there are at least two different binding sites and these may be similar to the two different isozymes already described in the rat,  $\alpha$  and  $\alpha$ -1.

We have also continued our work on the mechanisms of catecholamine retention in vesicles in situ. These synaptic vesicles are in adrenergic nerve terminals in slices of rat heart ventricle and have been studied for their release of stored tritiated NE. Ammonia not only neutralizes the acid Ph of the vesicle interior in the presence of ATP, but also releases amines present in vesicle ghosts. Thus ammonia has an inhibitory effect on storage by vesicles in situ. This alkalinizing action blocks ATP dependent uptake and releases NE. A series of such responses were compared with those of reserpine, which blocks uptake and releases NE, but has no effect on the electrochemical gradient of  $\text{H}^+$ . Ammonia predominantly released deaminated metabolites of NE from nerve terminals that were incubated in Krebs' ringer's buffer. These findings support our earlier work on the existence of a secretory mechanism that does not involve exocytosis. Secretion is mediated by transport of NE from intact vesicles attached to the axolemma. It appears that NE is passively retained, after uptake, by a pH gradient across the vesicle membrane. The acid pH of the vesicle keeps the amine in its permanent cationic form, awaiting depolarization and subsequent secretion.

III. Atrial natriuretic factor (ANF). Atrial natriuretic factor is secreted from atrial myocytes in response to various stimuli, including atrial stretch, tachycardia, pressor hormones, opiates, etc. In the past, we have presented evidence that the pituitary is involved in ANF release; i.e., in hypophysectomized animals, acute stimuli failed to cause an acute increase in plasma ANF. Attempts to find the substance in the pituitary that would reestablish responsiveness of ANF to acute stimuli led us to study more completely the responses of ANF to other forms of stimuli. We have developed a chronic stimulus for ANF release by creation of an aortocaval fistula, which causes high output heart failure and chronic volume overload in rats. While hypophysectomized rats had lower blood pressure, heart rate and right atrial pressure than control rats, both before and after aortocaval fistula, the increments in right atrial pressure were similar in both groups. ANF levels increased  $>200\%$  in both groups, and thus, in this form of stimulation, there was no evidence for a role of a hypophyseal factor. When rapid atrial pacing was performed for five



minutes in hypophysectomized rats, there was also a similar increase of plasma ANF in both operated and control animals. Again, there appeared to be no specific role for a hypophyseal factor. We then performed bilateral atrial appendectomy in rats and studied their response to various forms of stimuli, both acute and chronic. In this model, we found a differential response, i.e., the response to acute volume expansion was severely blunted, whereas the response to acute atrial pacing was normal. Thus it appears that hemodynamic factors are the important determinants of ANF release, and there is no evidence that a hypophyseal factor is required in any of the either acute or chronic forms of stimulation that we have studied. In addition, removal of the atrial appendages, does not preclude a normal ANF response to either acute or chronic stimuli.

In order to better understand the role of ANF in hypertension, we have studied the presence of ANF and angiotensin II receptors in both the central nervous system and several peripheral tissues in a number of hypertensive rat models. We found specific ANF binding sites on both stellate and celiac ganglia, the subfornical organ and choroid plexus, area postrema and pituitary of the brain, as well as the pelvis, medulla and outer cortex of the kidney. The number of ANF binding sites in areas of the brain that relate to blood pressure control was increased in the 2 kidney, 1 clip hypertensive rat, when compared to normotensive controls, but there were no differences noted in binding affinity. We have found a similar increase in the number of binding sites in the same brain areas in both SHR and DOCA-salt hypertensive rats when compared to their normotensive controls. Again, these have not been accompanied by any changes in binding affinity. It will be important to see if these changes in the number of binding sites are affected by normalization of the blood pressure in these hypertensive animals.

IV. Cellular mechanisms: We have been studying the cellular mechanisms of hypertension and atherosclerosis and their interactions in smooth muscle cells. We have shown that hypoxia accelerates cellular uptake of cholesterol, an initial step in atherogenesis, by cultured rat aorta smooth muscle cells (SMC). This is especially true when the SMC are cultured in hyperlipidemic serum. The increase is even greater when the study is performed under hypoxic conditions. In addition, the proliferation rate of SMC, prepared by the explant method, was greater for stroke prone spontaneously hypertensive rats (SHRsp) than for normotensive Wistar-Kyoto rats (WKR). Nitroprusside, a potent cyclic GMP stimulator, inhibited strongly the proliferation of SHRsp-SMC. DBcGMP and ANF (a guanylate cyclase stimulator) did not inhibit the proliferation of SMC. Phorbol ester pretreatment inhibited fetal calf serum induced DNA synthesis in quiescent SMC, and this effect was additive to the inhibitory effect of nitroprusside. These observations provide additional information





about the appropriate balance of the effects of c-AMP and c-GMP on the proliferation of SMC. We also found interesting differences in the effects of various substances on SMC, depending on how the cells were grown. SMC cultured on cover slips showed increased intracellular calcium to NE, but not to phenylephrine, and platelet derived growth factor (PDGF). Suspended SMC did not respond to NE, phenylephrine or PDGF. These results and others suggest that the receptors causing calcium influx into suspended SMC are damaged during the process of preparing the suspended cells. Thus growth on cover slips or other supporting media may be required for further evaluation of these cells and their responses.

V. Protein, aminoacids and diet: Hypertension responds better to weight reduction than it does to dietary sodium restriction. To test the possible mechanisms by which this occurs, we have begun a study in obese hypertensive patients and are monitoring the responses of their blood pressure and of various vasoactive substances to weight loss. Of 15 obese hypertensive patients who have entered into the study, the blood pressure has been normalized in half of the subjects with a 10-15% weight loss. It is still too early to determine which vasoactive substances may be involved in this process and will correlate best with weight reduction. From our studies in rats, we have demonstrated that SHRsp fed a low protein diet had higher blood pressure, greater incidence of stroke and greater cardiac hypertrophy than animals fed a normal protein diet. These effects are reversed by a high protein diet or a diet rich in methionine. We have recently studied the hemodynamic basis for this hypertension and have found that the relative cardiac output is the same in all groups regardless of protein intake, but the relative total peripheral resistance is significantly higher in animals fed a low protein diet. The reason for this effect of protein is unknown. However, we have conducted a study on the effects of bolus infusions of sulfur-containing aminoacids on blood pressure in several groups of rats. We have found that in SHR, acute bolus injections of sulfur aminoacids reduce the levels of blood pressure by 20-30%, heart rate by 10-15%, and total peripheral resistance by 20-35%. Cardiac output remained unchanged and plasma ANF increased three-fold. Administration of the same doses in pithed rats also reduced blood pressure by 30-50%. In normotensive WKR, we could observe the same effects on blood pressure only at much higher doses, i.e., two-fold more for taurine or cysteine sulfinic acid, and four-fold for methionine or penicillamine. These doses of sulfur-containing aminoacids also led to a reduction of blood pressure by a reduction of heart rate. Thus their action in lowering blood pressure came about by both a reduction of heart rate and of total peripheral resistance, but with an increase in stroke volume since cardiac output remained unchanged.

VI. Secretion and regulation of other factors: Additional work was



carried out to determine the role of the peptide, substance P (SP), as a neurotransmitter in the nervous system. Previous studies in our laboratory had demonstrated the nature of the interaction of this neuronal system with both the dopaminergic and serotonergic nervous systems. Current experiments were designed to further understand both the development of the system and its interaction with the endocrine system. Thus specific staining for SP receptors was found on certain neurons, certain nerve processes, and Purkinje cells, by light microscopy in different brain sections. These details were not observed previously in autoradiograms. This is especially true in the substantia nigra, where previous reports were negative for localization of SP receptors by autoradiography. Our new technique showed moderate staining of neurons in this area. Both SP and SP receptors were demonstrated in the mid-brain periaqueductal gray (PAG) area. In this area, SP may play an important role in pain mechanisms. In addition, a collaboration was begun with Genentech to screen SP clones from a rat hypothalamus cDNA library in an attempt to clone out the SP receptor.

Work has continued on the identification of the endogenous modulator (EM) of calcium channel activity that we isolated previously from whole rat brain. The active nitrendipine-displacing material has an apparent molecular weight of 1200 d, is heat-stable, soluble in water, methanol, or ethanol, and insoluble in chloroform or ether. Acid hydrolysis abolishes the nitrendipine-displacing activity and measurements of calcium current in isolated guinea pig ventricular cells showed that the material, when placed on the outside of the cell, strongly enhanced the high threshold calcium current. This activating effect was slowly reversible on washout and was not blocked by either beta- or alpha- adrenergic receptor antagonists. In contrast, in neuroblastoma cells, addition of EM caused a marked suppression of the low and high threshold currents within a few milliseconds. Acid-hydrolyzed EM failed to suppress calcium current in neuroblastoma cells. Ordinary sequencing activities have failed to identify this material, as have attempts at mass fragmentography.

We have continued to pursue the role of prostaglandin  $\text{PGI}_2$  in the renal vasodilatation induced by acetylcholine (ACh) in indomethacin (Indo)-treated dogs. Renal arterial infusions of ACh in control dogs produced an increase in sodium excretion and renal plasma flow, without a change in glomerular filtration rate (GFR) or renin secretory rate (RSR). In dogs pretreated with Indo, an inhibitor of prostaglandin synthetase, renal arterial infusion of ACh produced an increase and then a decline in sodium excretion, accompanied by a progressive fall in GFR and renal plasma flow (RPF), and a progressive rise in RSR. Renal arterial infusion of  $\text{PGI}_2$  before and during the infusion of ACh restored the natriuretic and vasodilatory responses to ACh in Indo-treated dogs. The data strongly suggest that Indo



shortens the natriuretic and vasodilator response to ACh, by inhibiting the synthesis of prostaglandins, possibly  $\text{PGI}_2$ , and that  $\text{PGI}_2$  may play a role in the renal vasodilatory effect of ACh.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01996-03 HE

## PERIOD COVERED

Oct. 1, 1987 to Sept. 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Modifications of ANF and AII receptors in hypertension.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Carmen Gonzalez Visiting Fellow HE NHLBI

Others: Harry R. Keiser Chief HE NHLBI  
Juan M. Saavedra Unit Chief CS NIMH

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Hypertension-Endocrine Branch

## SECTION

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2.0

## PROFESSIONAL:

2.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have been studying the presence of receptors for atrial natriuretic factor (ANF) and Angiotensin II (AII) in the central nervous system (CNS), and in several peripheral tissues in a high renin hypertensive rat model (2 kidney, 1 clip).

We found specific ANF and AII binding on several areas of the brain that are associated with circulatory control.

We have found an increase in the number of ANF binding sites in several circumventricular areas and in the choroid plexus. These results are similar to other earlier findings in spontaneously hypertensive rats and DOCA-salt hypertensive rats.

In peripheral organs, we have found a difference in the number of binding sites for Angiotensin converting enzyme (ACE) in renal tubule.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03559-02 HE

## PERIOD COVERED

Oct. 1, 1987 to Sept. 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Catechols and sympathoadrenomedullary function in health and disease

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

David S. Goldstein, Senior Investigator, HE, NHLBI; John Bacher, VRB, DRS, NIH; John E. Brush, Jr, CB, NHLBI; Anna Deka-Starosta, Visiting Fellow, DIR, NINCDS; Graeme Eisenhofer, Visiting Fellow, DIR, NINCDS; Ronald Finn, Nuclear Medicine Dept. CC, NIH; Moshe Garty, Visiting Associate, DIR, NINCDS; John R. Gill, Jr., Senior Investigator, HE, NHLBI; David Hovevey-Sion, Visiting Fellow, DIR, NINCDS; Harry R. Keiser, Chief, HE, NHLBI; Kenneth Kirk, LC, NIDDK; Irwin J. Kopin, Scientific Director, DIR, NINCDS; Robert Miletich, Medical Staff Fellow, NINCDS; Robin Stull, Chemist, HE, NHLBI; Katalin Szemerédi, Visiting Fellow, DIR, NINCDS; Zofia Zukowska-Grojec, Guest Worker, HE, NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Hypertension-Endocrine Branch

## SECTION

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2.0

## PROFESSIONAL:

2.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Physiological changes in directly recorded renal sympathoneural activity were related to changes in renal NE spillover into the bloodstream in rats, and physiological changes in skeletal muscle sympathoneural activity were related to changes in antecubital venous NE in humans. Juvenile spontaneously hypertensive rats had excessive sympathoadrenomedullary responsiveness to yohimbine without increases in corticotropin. About 1/3 of young patients with essential hypertension also had excessive sympathoadrenomedullary responsiveness to yohimbine with normal corticotropin responses. Young hypertensives had excessive sympathoadrenomedullary and pressor responses to playing a video game, with normal increments in mean arterial pressure for given increments in total body NE spillover. Patients with hypertrophic cardiomyopathy had defective neuronal uptake of NE. A method for in vivo measurement of reuptake of endogenously released NE was introduced, based on simultaneous measurements of NE and dihydroxyphenylglycol (DHPG). Regional release of dihydroxyphenylalanine (DOPA) indicated regional catecholamine biosynthesis. DOPA appeared to be a natriuretic neurohormone derived from sympathetic nerve endings and the brain. In dogs, all of urinary dopamine excretion was derived from plasma DOPA. Dietary salt loading increased urinary dopamine excretion by increasing DOPA delivery to renal uptake sites in humans. Salt-sensitive hypertensives appeared to have defective conversion of DOPA to dopamine in the kidney. Pituitary-adrenocortical and sympathoadrenomedullary system activities interacted in a compensatory manner to maintain homeostasis. PET scanning after injection of positron-emitting fluorodopamine successfully visualized tissue sympathetic innervation and function.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03563-02 HE

PERIOD COVERED

Oct. 1, 1987 to Sept. 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Endogenous calcium channel modulator

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Ingeborg Hanbauer	Pharmacologist	HE NHLBI
Others:	Mariagrazia Grilli	Visiting Fellow	HE NHLBI
	Enrico Sanna	Visiting Fellow	HE NHLBI
	Gil Wright, Jr.	Research Chemist	HE NHLBI

COOPERATING UNITS (if any)

Martin Morad Professor, Dept. of Physiology, Univ. of Pennsylvania, Phila. PA.

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

Radiolabelled dihydropyridines were used as a probe to monitor for material with high affinity for dihydropyridine recognition sites. Material with an apparent molecular weight of 1100 daltons was isolated from whole rat brain, and was purified by HPLC technique. Studies on 3-H-nitrendipine binding in saturating conditions in the absence of the presence of this endogenous modulator showed that it was not competing for dihydropyridine recognition sites but indicated an allosteric modulation of these sites. The endogenous modulator inhibited reversibly calcium accumulation elicited by veratridine in cerebellar granule cells in tissue culture, and consequently blocked increase of cyclic GMP formation and protein kinase C activation in these cells. The role of the endogenous modulator on calcium channel activity was further substantiated in neuroblastoma cells (NIE-115) using whole cell patch clamp technique. Both low and high threshold types of calcium channels that occur in these cells were suppressed by the endogenous modulator at all potentials tested.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03564-02 HE

## PERIOD COVERED

Oct. 1, 1987 to Sept. 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulatory mechanisms for adrenal chromaffin cell secretion

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Ingeborg Hanbauer

Pharmacologist

HE NHLBI

## COOPERATING UNITS (if any)

Fidia-Georgetown Institute for Neuroscience, Washington, DC (A. Guidotti)

## LAB/BRANCH

Hypertension-Endocrine Branch

## SECTION

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The role of GABA receptors in the function of adrenal chromaffin cells was studied in situ by autoperfusion of canine adrenal gland. GABA-A receptor agonists increased the resting outflow of catecholamine and met-enkephalin-like peptides in the adrenal venous blood, while GABA-B receptor agonists had no effect. Denervation of the adrenal gland two weeks before the experiment failed to prevent the effect of GABA-A receptor agonists indicating that GABA-A receptors are located on chromaffin cells. When the release of catecholamines and met-enkephalin-like peptides was elicited by electrical stimulation of the splanchnic nerve GABA-A receptor agonists attenuated this increase while GABA-A-receptor antagonists facilitated it. Direct stimulation of GABA-A receptors located on chromaffin cell membranes causes bursts of Cl<sup>-</sup> channel opening resulting in depolarization of chromaffin cell membranes and thereby facilitating the spontaneous release of neurotransmitters. It is proposed that this type of depolarization involving bursts of Cl<sup>-</sup> channel opening may obtund the depolarizing effect of nicotinic receptor stimulation. Hence in the adrenal medulla GABA can be viewed as a primary transmitter that modifies the effect of nicotinic receptor stimulation by acting on a different transducer system, presumably the Cl<sup>-</sup> channel.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03565-02 HE

## PERIOD COVERED

Oct. 1, 1987 to Sept. 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Biosynthesis, distribution and biological function of substance P and its receptors

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Mei-Lie Swenberg	Research Chemist	HE NHLBI
Others:	Rita Liu	Asst. Professor	Georgetown Univ.
	Bernard Malfroy	Scientist	Genentech, Inc.
	Brian M. Martin	Research Scientist	ADAMHA

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Hypertension-Endocrine Branch

## SECTION

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2.0

## PROFESSIONAL:

2.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Antibodies against SP receptors from rat olfactory bulb (Ant-Rc-olf) and intestinal mucosa (Ant-Rc-Int) prepared by immunizing New Zealand white rabbits with the purified receptors were proved active in binding to fixed tissue sections and the isolated receptor proteins by immunocytochemical localization and western blot respectively.

A project on screening SP clones from rat hypothalamus cDNA library has been initiated in April 1988 in collaboration with Genentech, Inc.

Project on amino acid sequence of SP receptor protein has been carried out in collaboration with Dr. B. Martin. The results will be obtained as soon as the problem of transferring the purified protein from the acrylamide gel to the immobilon (polyvinylidene difluoride, PDrF) membrane is resolved.

Reports of the evidence that SP, the putative neurotransmitter, is distributed in the midbrain periaqueductal gray (PAG), suggest the possibility of SP involvement in the pain processing mechanism in PAG. Therefore the localization of substance P (SP) and SP binding sites were conducted on the periaqueductal gray (PAG) of the rat to examine their relationship. Autoradiograms and immunocytochemical localization revealed an uneven distribution of both specific binding sites and SP-immunoreactivity (SP-ir) respectively.

521





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03567-01 HE

## PERIOD COVERED

Oct. 1, 1987 to Sept. 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of the dopamine reuptake system

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Ingeborg Hanbauer

Pharmacologist

HE NHLBI

Others: Mariagrazia Grilli

Visiting Scientist

HE NHLBI

Enrico Sanna

Visiting Fellow

HE NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Hypertension-Endocrine Branch

## SECTION

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

.5

## PROFESSIONAL:

.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Na<sup>+</sup>-dependent binding sites for 3-H-mazindol or 3-H-cocaine are specifically located in dopaminergic nerve terminals of caudate nuclei and constitute a molecular component of the dopamine uptake domain. The Na<sup>+</sup>-dependent recognition sites for 3-H-cocaine in striatal synaptosomal membranes appear to be regulated by: (1) Receptor-receptor interaction involving corticofugal glutamatergic nerve endings; and by (2) GABA-modulin, a neuromodulatory protein present in synaptosomal membranes. Destruction of cortico-striatal glutamatergic nerve fibers increased the density of 3-H-cocaine and 3-H-mazindol binding sites in striatal synaptosomal membranes and increased the efficacy of cocaine or mazindol to inhibit 3-H-dopamine uptake in striatal slices. GABA-modulin decreased the number of the Na<sup>+</sup>-dependent binding sites of 3-H-cocaine indicating an allosteric receptor modulation.

525







## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03569-01 HE

## PERIOD COVERED

Oct. 1, 1987 to Sept. 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mechanisms of blood pressure control in obese hypertensive subjects.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	James C.M. Chan	Special Volunteer	HE NHLBI
Others:	Harry R. Keiser	Chief	HE NHLBI
	Frederic Sax	Senior Staff Fellow	CB NHLBI
	Antonio L. Bartorelli	Guest Worker	CB NHLBI
	Gloria J. Stables	Dietitian	NUTR CC
	Josephine E. Jacobs	Bio Lab Tech	HE NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Hypertension-Endocrine Branch

## SECTION

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Obese (120-150% of ideal body weight) hypertensive adults will be placed on a weight reduction diet in the outpatient clinic and followed weekly. Blood samples and 24 hour collections of urine, for evaluation of a number of systems that may be involved in the decrease in blood pressure associated with weight loss, will be obtained at the following times: (a) before the diet begins; (b) when blood pressure has decreased by 5-10 mmHg in association with mild weight loss and (c) when the patient is close to ideal body weight. After the last collection, the patient will remain on the same diet for another week and will take sodium chloride and calcium carbonate tablets in amounts needed to equal the original intake of sodium and calcium. Blood pressure will be monitored to see if there is any reversal of the blood pressure reduction.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03570-01 HE

## PERIOD COVERED

Oct. 1, 1987 to Sept. 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Role of prostaglandin I<sub>2</sub> in the renal vasodilatation induced by acetylcholine.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	John Yun	Guest Worker	HE NHLBI
Others:	John R. Gill, Jr.	Senior Investigator	HE NHLBI
	Harry R. Keiser	Chief	HE NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Hypertension-Endocrine Branch

## SECTION

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The role of prostaglandin I<sub>2</sub> (PGI<sub>2</sub>) in the renal vasodilatation induced by acetylcholine (ACh) was examined in indomethacin (Indo)treated dogs receiving an infusion of PGI<sub>2</sub>. Renal arterial infusion of ACh (40µg/min) in control dogs produced an increase in sodium excretion and renal plasma flow (RPF) without a change in glomerular filtration rate (GFR) or renin secretory rate (RSR). In dogs pretreated with indomethacin (Indo), an inhibitor of prostaglandin synthesis, renal arterial infusion of ACh produced an increase and then a decline in sodium excretion accompanied by a progressive fall in GFR and RPF and a progressive rise in RSR. Renal arterial infusion of PGI<sub>2</sub> (0.2µg/min) before and during the infusion of ACh restored the natriuretic and vasodilatory response to ACh in Indo-treated dogs. The data suggest that Indo shortens the natriuretic and vasodilatory response to ACh by inhibiting synthesis of prostaglandins, possibly PGI<sub>2</sub>, and that PGI<sub>2</sub> may play a role in the renal vasodilatory effect of ACh.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03571-01 HE

## PERIOD COVERED

Oct. 1, 1988 to Sept. 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Hemodynamics and dietary protein in hypertension

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Martina Diolulu	Guest Researcher	HE NHLBI
Others:	Stergios Kapoulas	Visiting Associate	HE NHLBI
	Harry R. Keiser	Chief	HE NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Hypertension-Endocrine Branch

## SECTION

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Hemodynamic variables were evaluated in 4-month-old normotensive Wistar Kyoto rats (WKY) and spontaneously hypertensive stroke-prone rats (SHRSP) that had been maintained for 3 months on one of four experimental diets: standard (STD; 24% crude protein), low protein (LP; 19% crude protein), high protein (HP; 32% crude protein), or high methionine (Met; 1.9% methionine). WKY/STD and SHRSP/LP had similar body weights (BW) that were significantly higher than those of SHRSP/STD, SHRSP/HP and SHRSP/Met. The heart weights (HW) of SHRSP/LP were significantly higher than those of the other experimental groups. The HW/BW ratio for WKY/STD was significantly less than that for any of the SHRSP groups, while the HW/BW ratio for SHRSP/LP was significantly higher than that for SHRSP/HP and SHRSP/Met.

The systolic blood pressure ( $BP_s$ ) of all SHRSP groups was significantly higher than that of WKY/STD, while the  $BP_s$  of SHRSP/STD was significantly higher than that of SHRSP/Met, SHRSP/HE and SHRSP/LP. The diastolic blood pressures ( $BP_d$ ) of WKY/STD, SHRSP/Met and SHRSP/HP were the same but significantly lower than those of SHRSP/STD and SHRSP/LP. WKY/STD and SHRSP/Met groups had similar mean arterial pressures (MAP) that were significantly lower than those of SHRSP/HP, SHRSP/STD and SHRSP/LP. The relative total peripheral resistance (TPR) in WKY/STD, SHRSP/Met and SHRSP/HP was similar but significantly lower than that of SHRSP/STD and SHRSP/LP. The results of these experiments indicate that the increased MAP in SHRSP fed either a standard rat diet or a low protein diet was due to increased TPR, while a high protein diet led to nearly normal MAP due to a reduction in TPR.

538



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03572-01 HE

## PERIOD COVERED

Oct. 1, 1987 to Sept. 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Role of vesicular pH in storage of NE in adrenergic synaptic vesicles

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: D.F. Bogdanski

Pharmacologist

HE NHLBI

Others:

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Hypertension-Endocrine Branch

## SECTION

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This laboratory has studied the storage of norepinephrine (NE) in synaptic vesicles in the axoplasm within adrenergic nerve terminals (in situ). Storage depended upon energy utilizing reactions involving ATP at the membranes of vesicles (Bogdanski and Blaszkowski, 1973). The results of other studies have shown that vesicles in situ respond to known inhibitors of the uptake dependent upon  $Mg^{++}$ -ATPase activity in the membranes of isolated vesicles (Bogdanski, 1982; 1983; 1986; 1988). In isolated vesicles, the activity of  $Mg^{++}$ -ATPase drives protein transport to generate electrochemical gradients of protons across the vesicle membrane. These gradients drive the transport of NE. Biochemical reagents which block or dissipate the gradients of  $H^+$  block uptake and, generally, release NE from vesicles in situ.

The past years' work has dealt primarily with the inhibitory effect of ammonia on storage by vesicles in situ. This alkalinizing reagent for vesicles blocks ATP-dependent uptake, and releases NE from vesicles. A series of such responses were compared with those of reserpine, which blocks uptake and releases NE but has no effect on electrochemical gradients of  $H^+$ .



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03573-01 HE

## PERIOD COVERED

Oct. 1, 1987 to Sept. 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies of atrial natriuretic factor release in rats

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Aaron Hoffman

Visiting Associate

HE NHLBI

Others: Harry R. Keiser

Chief

HE NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Hypertension-Endocrine Branch

## SECTION

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

0.3

## PROFESSIONAL:

0.3

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Atrial natriuretic factor is secreted from atrial myocytes in response to various stimuli: atrial stretch, tachycardia, pressor hormones, opiates, etc. In our studies we addressed 2 questions: 1) Whether the hypophysis is involved in ANF release, as suggested by some investigators, 2) Whether atrial appendectomy, thus removing the main source of ANF, alters its release to different stimuli.

We used hypophysectomized rats and applied 1) a chronic stimulus for ANF release by placing an aortocaval fistula which causes high output heart failure and chronic volume overload, and 2) an acute stimulus in the form of rapid atrial pacing of 500 beats/minute for 5 minutes. In both types of stimuli we found a marked increase of plasma ANF to a similar level as in control rats.

In conscious, chronic atrial-appendectomized rats we applied 1) acute volume expansion, 2) salt load, and 3) norepinephrine as acute stimuli as well as an aortocaval fistula for a chronic stimulus. We found a differential response: the one to acute volume expansion was severely blunted, as reported in the literature, whereas the other acute stimuli and the chronic stimulus elicited a normal increase in plasma ANF.

We conclude that: 1) Hemodynamic factors are the important determinants of ANF release 2) no hypophyseal factor is required and 3) removing the atrial appendages does not preclude a normal ANF response to different stimuli.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 HL 03574-01 HE
PERIOD COVERED Oct. 1, 1987 to Sept. 30, 1988		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <b>Cellular mechanisms of hypertension and atherosclerosis</b>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI: Seiichi Shimizu	Visiting Associate	HE NHLBI
Others: Margaret Hill	Medical Tech	HE NHLBI
Reuben Brown	Phys. Sci. Tech	HE NHLBI
Harry R. Keiser	Chief	HE NHLBI
COOPERATING UNITS (if any)  None		
LAB/BRANCH Hypertension-Endocrine Branch		
SECTION		
INSTITUTE AND LOCATION NHLBI, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS: 1.0	PROFESSIONAL: 1.0	OTHER:
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>             Total cholesterol levels in SMC cultured with hyperlipidemic serum, increased more under hypoxic conditions than under normal conditions. No difference was noted in cholesterol synthesis from acetic acid. TG levels in SMC were inhibited markedly under hypoxic conditions. TG synthesis induced by serum in the hypoxic cells was decreased when compared with control cells. Thus hypoxic conditions accelerate cellular uptake of cholesterol, an initial step in atherogenesis.           </p> <p>             Proliferation rate of SMC prepared by the explant method was greater for SHRsp than WKY. SHRsp-SMC prepared by the enzyme method proliferated at the same rate as those prepared by the explant method. However, proliferation rate of WKY-SMC by enzyme method was extremely low. Nitroprusside inhibited strongly the proliferation of SHRsp-SMC. DBcGMP and ANP did not inhibit the proliferation of SMC. Methylene blue did not interrupt the inhibitory effect of Nitroprusside. It is suggested that Nitroprusside has an unknown biological action. TPA inhibited fetal calf serum induced DNA synthesis in quiescent SMC. The TPA effect was additive to the inhibitory effect of Nitroprusside.           </p> <p>             SMC cultured on cover slips showed increased intracellular <math>[Ca^{2+}]_i</math> to norepinephrine but not Phenylephrine and PDGF. Suspended SMC did not respond against NE, Phenylephrine and PDGF. In suspended SMC and SMC cultured on cover slip, angiotensin II caused increases in <math>[Ca^{2+}]_i</math>. A specific inhibitor of AGII, <math>[Sar^1, Ile^8]AGII</math>, blocked increases in Fura fluorescence elicited by AGII.           </p>		





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03575-01 HE

## PERIOD COVERED

Oct. 1, 1987 to Sept. 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Role of the  $\text{Na}^+\text{K}^+\text{ATPase}$  on catecholamine secretion in the chromaffin cell

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Carmen Gonzalez

Visiting Fellow

HE NHLBI

Other: Harry R. Keiser

Chief

HE NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Hypertension-Endocrine Branch

## SECTION

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The presence of the enzyme  $\text{Na}^+\text{K}^+\text{ATPase}$  in the bovine adrenal gland has been studied using 3 different approaches:  $^3\text{H}$ -ouabain binding, catecholamine (CA) secretion evoked by cardiac glycosides and  $^{86}\text{Rb}$  uptake sensitive to cardiac glycosides.

We found only one homogeneous population of  $^3\text{H}$ -ouabain binding sites likely located on the plasmatic membrane of chromaffin cells with a  $K_d=13.6\pm1.2\text{nM}$  and a  $B_{\text{max}}=3.15\pm0.21\text{pmol/mg}$  protein. The  $^3\text{H}$ -ouabain binding was specific and could be displaced by other cardiac glycosides with an order of potency of ouabain > acetyldigoxin > digitoxin.

Blockade of  $\text{Na}^+\text{K}^+\text{ATPase}$  with cardiac glycosides induced CA release from isolated bovine chromaffin cells. This CA release was both  $\text{Ca}^{2+}$  and temperature dependent. The order of potency for cardiac glycosides was ouabain > strophanthidin > digitoxin.

We also tried to correlate the CA release induced by cardiac glycosides to the  $\text{Na}^+\text{K}^+\text{ATPase}$  activity measured as  $^{86}\text{Rb}$  or  $^{42}\text{K}^+$  uptake in isolated chromaffin cells.  $^{86}\text{Rb}$  uptake reached a plateau at 60 minutes. A similar course was obtained with  $^{42}\text{K}^+$ .  $^{86}\text{Rb}^+$  uptake was blocked in a dose-dependent manner by cardiac glycosides, being ouabain the most potent drug.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03576-01 HE

## PERIOD COVERED

Oct. 1, 1987 to Sept. 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

DOPA metabolism in salt-resistant and salt-sensitive hypertension.

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: John R. Gill, Jr. Senior Investigator HE NHLBI

Others: David Goldstein Senior Investigator HE NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Hypertension-Endocrine Branch

## SECTION

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

## PROFESSIONAL:

## OTHER:

1.0

1.0

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In a previous study in normal subjects an increase in sodium intake from 9 mEq/day to 249 mEq/day was associated with an increase in urinary DOPA without a change in plasma DOPA. These observations suggested that sodium administration increased the delivery of DOPA to the kidney. Since the excretion of DOPA, the precursor of dopamine, paralleled the excretion of dopamine, the increased delivery of DOPA and its increased uptake by the kidney presumably was responsible for the increase in dopamine excretion that accompanied the increase in sodium intake. Since dopamine excretion is abnormal in both salt-resistant (SR) and salt-sensitive (SS) hypertension, the possibility that abnormalities in DOPA metabolism were responsible was studied. On a sodium intake of 9 mEq/day DOPA excretion was twice normal in both SR and SS and dopamine excretion in SR was higher ( $P < 0.01$ ) than normal. When sodium intake was increased DOPA excretion increased in SR and SS by 47% and 96%, respectively, (versus 66% in normal subjects) but urinary dopamine increased by only 18% and 15%, respectively, (versus 49% in normal subjects). Plasma DOPA was normal and did not change. The results indicate that the formation of DOPA is increased in SR and SS hypertensives and this could explain the supernormal formation of dopamine in SR. The results also indicate that the uptake of DOPA by the kidney or its conversion to dopamine is impaired in SS.

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ANNUAL REPORT OF THE  
LABORATORY OF KIDNEY AND ELECTROLYTE METABOLISM  
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE  
October 1, 1987 to September 30, 1988

Our continuing goal is to analyze the function of the kidney as a basis for understanding its pathophysiology and treating its disorders. Since the formation of urine depends upon the transport of water and solutes by kidney tubules, understanding renal function requires analysis of these cellular processes and of their integration in the kidney. Therefore, we are studying transport by cells in general and kidney cells in particular, as well as the mechanisms, hormonal and other, that support and control transport and metabolism.

Isolated segments of renal tubules.

In order to understand kidneys at a cellular and molecular level the functions of the different types of epithelial cells must be identified. Progress in this direction has relied heavily on the direct study of individual nephron segments. Each nephron segment has a different cell morphology and function. An important method (which originated in this laboratory) for directly studying the nephron segments is to dissect them and perfuse them individually in vitro. The findings of Knepper and his colleagues during the past year using this method are as follows:

Nonoguchi, Sands and Knepper studied the effect of atrial natriuretic factor (ANF) on water and urea transport in the inner medullary collecting duct (IMCD). ANF decreased vasopressin-stimulated osmotic water permeability ( $P_f$ ). Half maximal inhibition occurred at a concentration similar to that measured in rat systemic plasma during extracellular fluid volume expansion. ANF had no effect in the absence of vasopressin, and had no effect on vasopressin-stimulated urea permeability. ANF also had no effect on vasopressin-stimulated cyclic AMP (cAMP) accumulation and inhibited cAMP-stimulated  $P_f$ . Thus, ANF inhibits a "post-cyclic AMP" step in the chain of events that couple the binding of vasopressin to its receptor and activation or insertion of water channels in the apical membrane.

Nonoguchi, Sands and Knepper found that ANF inhibits NaCl and fluid absorption in rat cortical collecting ducts (CCDs). In the absence of vasopressin, ANF inhibited the net Na<sup>+</sup> flux by 90%. In the presence of vasopressin, spontaneous fluid absorption was inhibited by 50-80%. ANF inhibited vasopressin-stimulated osmotic water permeability, but only by about 11%. Cyclic GMP also inhibited vasopressin-stimulated spontaneous fluid absorption, indicating that cyclic GMP may be the intracellular messenger in the action of ANF on the CCD. ANF-mediated inhibition of NaCl and fluid absorption in the cortical collecting duct can account for part of the increase in NaCl and water excretion seen in animals infused with ANF.

Mejia, Sands and Knepper carried out mathematical modelling of ANF action in the kidney. A model describing NaCl, urea, and water transport in the rat kidney was developed and applied to an examination of possible sites of ANF action in



the kidney. The model consists of a large array of steady state balance equations that account for mass balance in each nephron segment and in the interstitial-vascular space. The system of equations is solved using a Cray XMP computer. Mathematical simulations were carried out to examine quantitatively the possible actions of ANF in the kidney. They used the model to determine hypothetical effects of ANF-mediated increases in glomerular filtration rate (GFR) and of ANF-mediated decreases in collecting duct NaCl absorption. The results showed that both an increase in GFR and a decrease in collecting duct NaCl transport can be predicted to increase NaCl and water excretion as observed in vivo. However, only direct inhibition of NaCl transport in the collecting duct could account for observed increases in urinary NaCl concentration to levels above that present in plasma. These studies support the view that ANF-mediated inhibition of NaCl absorption in the collecting duct could play an important role in the natriuretic response to ANF.

Wall, Sands, and Knepper studied acid-base transport in rat terminal IMCDs. Significant rates of bicarbonate absorption and ammonium secretion were found in tubules from untreated rats in the absence of imposed driving forces for passive transport of these ions. Systemic ammonium chloride administration and in vitro deoxycorticosterone increased the rate of bicarbonate absorption. Addition of vasopressin to the peritubular bath also increased the rate of bicarbonate absorption.

Sands and Knepper found evidence that urea and water transport are independent of each other in rat terminal IMCDs. Two methods were devised to measure the reflection coefficient for urea. Both approaches gave values not significantly different from unity, indicating an absence of coupling between water and urea transport. Thus, although both water and urea transport across the IMCD cells are stimulated by vasopressin, the two pathways are distinct and independent.

Chou and Knepper studied the mechanism of urea transport in rat terminal IMCDs. The major question was whether the urea transport mechanism in the rat terminal IMCD has the characteristics of a facilitated transport pathway. The apparent urea permeability was found to decrease as absolute urea concentration increased, consistent with saturation of a carrier or channel. The urea analogues thiourea, methylurea, and acetamide inhibited urea permeation. Phloretin, an inhibitor of carrier mediated urea transport in red blood cells and toad urinary bladders, markedly inhibited urea transport, but had no effect on osmotic water permeability. The observed properties indicate that urea transport across the rat IMCD occurs via a specialized carrier or channel.

Terada and Knepper determined the distribution of Na-K-ATPase activity along the rat IMCD. The highest activities were found in the initial 25% of the IMCD. Lower, but significant, levels were measured in the terminal 75% of the IMCD. In vivo deoxycorticosterone administration and dietary NaCl restriction increased Na-K-ATPase activity throughout the IMCD.

Packer, Sands and Knepper studied vasopressin effects on chloride transport across the rabbit papillary surface epithelium. Chloride permeability was measured in isolated papillary surface epithelial sheets mounted in a small Ussing





chamber. Vasopressin added to the basolateral bathing solution reversibly decreased chloride permeability by about one-third and increased the transepithelial resistance. The osmotic water permeability was low and was not affected by vasopressin. Increased chloride permeability when vasopressin is removed may contribute to the partial dissipation of the medullary NaCl gradient that occurs during water diuresis.

#### Transport in model planar epithelia.

The transporters in some planar epithelia such as toad bladders, toad skins, and Necturus gall bladders are similar to those in parts of the nephron. These planar epithelia are easier to manipulate than individual nephrons, making them valuable models for studying the transporters. Spring and his colleagues have been studying solute and water transport by Necturus gall bladders and toad skins. They developed and used a combination of light microscopic, video, computer, and electrophysiologic methods to study cell volume and intracellular ions.

Garvin and Spring investigated the regulation of intracellular Na and Cl in Necturus gallbladder by the use of ion sensitive and voltage sensitive microelectrodes. They found that Cl exit from the cell involves Na and  $\text{HCO}_3$  and is probably due to a basolateral cotransport system. Such a cotransporter has been identified in many epithelial cells and seems to play a significant role in both transepithelial transport and in the regulation of cell pH.

Lowy and Spring are developing quantitative techniques for fluorescence microscopy of living cells. They are utilizing a new illumination and detection system developed by Spring and Smith for the study of living cells stained with fluorescent indicator dyes. They are also visualizing single virus particles, the VSV virus, invading renal epithelial cells. These approaches are designed to validate in living cells previous measurements on test targets and to extend the techniques to new areas of investigation.

Furlong and Spring are studying the ionic basis of cellular volume regulation after decreases in the osmolality of the bathing solution. They have shown that gallbladder cells regulate their volume by increasing the exit of K and Cl from their basolateral surfaces by opening individual channels for each ion. They are investigating the effects of a variety of inhibitors and drugs on the ability of the cells to readjust their volume after an osmotic challenge.

#### Cell culture of epithelia.

Although the technique of perfusing kidney tubules in vitro has provided an overall description of their transport properties, it has been difficult to extend the studies to subcellular and molecular levels. Chemical and physical methods for studying transport require much larger amounts of homogeneous tissue than are present in single tubules. Handler, Burg and their colleagues have been using cultures of renal epithelial cells to overcome this difficulty. In addition, epithelia in culture can be readily maintained for prolonged periods of time under conditions not obtainable in intact tissues, and the cultures are



more amenable to study by number of the techniques of cell and molecular biology.

Handler and his associates are applying the techniques of somatic cell genetics to study sodium-coupled phosphate transport in kidney derived cells in continuous culture. Cells are grown in medium with exceedingly low concentrations of inorganic phosphate and no other source of phosphorus. Since phosphorus is essential for growth, cells that grow in the low concentration of phosphate must alter their metabolism or transport of phosphate. The rate of sodium-coupled phosphate uptake in cells that grow in medium containing .01 mM phosphate is 8 to 20 times the rate in cells grown in standard medium containing 1.0 mM phosphate. There is no change in  $K_m$ . The cells will be studied for evidence of an increase in one or a few membrane proteins, possibly the transporter, and for amplification of the gene for the transporter. A positive result in either test would facilitate cloning the gene for the transporter.

### Osmolytes.

Bacterial, plant, and invertebrate animal cells are known to accumulate compatible, osmotically active, organic intracellular solutes when their environment became hyperosmotic. These "osmolytes" help maintain the intracellular milieu because they do not perturb vital intracellular macromolecules, in contrast to sodium and potassium salts which, in abnormally high concentrations, do perturb macromolecules. Most mammalian tissues are not normally hyperosmotic and presumably do not express osmolytes. The exception is the renal inner medulla which is hyperosmotic because of the renal concentrating mechanism. In previous studies Burg and his colleagues identified the principal osmolytes in rat and rabbit inner medullas as sorbitol, inositol, glycerophosphorylcholine, and betaine.

Yancey and Burg studied osmolytes in intact renal medullas of rabbits that were antidiuretic following two days of water deprivation or were diuretic from drinking more water than they normally do because their water was sweetened with sucrose for five days. There were gradients of sorbitol, betaine, and GPC, with levels rising towards the tip of the renal papilla. These solutes were absent from the cortex. Low levels of betaine and GPC, but not sorbitol were present in the outer medulla. Inositol was higher in the outer medulla than in the inner medulla, and was present in significant quantities in the cortex. The levels of all osmolytes were lower during diuresis, but the gradients were qualitatively the same.

Bagnasco, Uchida, Balaban, Kador and Burg previously found that sorbitol accumulates in GRB-PAP1 cells by synthesis from glucose, catalyzed by aldose reductase. Sorbitol accumulation and aldose reductase activity increase greatly when medium osmolality is elevated. Bedford, Bagnasco, Kador, Harris, and Burg observed that the additional aldose reductase activity is explained by an increase in aldose reductase protein, which may exceed 10% of cell protein in induced cells. They purified the enzyme and raised antiserum against it.



It seemed likely that increased translation is a likely explanation for the increase in aldose reductase protein. In order to test this directly Moriyama, Garcia-Perez, and Burg have set up an immunoprecipitation method. The initial studies, using pulses of  $^{35}\text{S}$ -methionine, demonstrate a much greater rate of synthesis of aldose reductase protein in cells grown in hyperosmotic medium.

Uchida, Murphy, and Burg found that induction of aldose reductase by hyperosmolality in GRB-PAP1 cells correlates with increased intracellular ionic strength and not with cell volume.

In order to determine whether transcription of aldose reductase increases during its induction, Garcia-Perez, Martin, Murphy, Uchida, Handler, and Burg have cloned cDNA for aldose reductase from induced GRB-PAP1 cells, and are in the process of sequencing it. In the initial studies using a RNA probe, prepared from the cDNA, the abundance of aldose reductase mRNA is greatly increased in cells grown in media with a high osmolality.

The osmoregulatory changes in aldose reductase protein in GRB-PAP1 cells are slow. Three to four days are required for full induction by hyperosmolality, and even longer for down regulation on return to lower osmolality. Bag-nasco, Murphy and Burg found that rapid osmoregulation of intracellular sorbitol concentration occurs by flux of sorbitol from the cells to the medium. Siebens and Spring observed that the increased flux is due to increased sorbitol permeability. Sorbitol permeability of the cells is low in hyperosmotic medium and increases greatly within seconds of lowering the osmolality. This is apparently a form of volume regulatory decrease. The increased permeability seems to be relatively specific. Siebens and Spring are attempting to determine the mechanism of the change in permeability.

In order to relate the control of sorbitol accumulation in vivo to the findings in tissue culture, Cowley and Burg are decreasing rat renal medullary extracellular NaCl concentration by chronic administration of furosemide or increasing it by dehydration. They are measuring aldose reductase protein by Western analysis and mRNA by Northern analysis. The initial measurements show that both are higher in the inner medullas of antidiuretic rats than following diuresis.

Nakanishi, Turner and Burg found that inositol accumulates in MDCK cells when medium osmolality is increased. The mechanism is increased transport into the cells from the medium. No accumulation occurs unless inositol is present in the medium. The transport is sodium-dependent and is inhibited by phlorizin.  $V_{\text{max}}$  increases, but  $K_m$  does not change.

In order to characterize the inositol transporter, Moo, Handler, and Burg are attempting to clone its cDNA by expression of mRNA in frog oocytes. Using sucrose gradient centrifugation, they have identified in the mRNA from induced cells fractions that increase sodium-dependent  $^3\text{H}$ -inositol uptake into frog oocytes. They will prepare a cDNA library from the most active mRNA fractions and use the oocyte expression system to screen mRNA transcribed from the cDNA clones.



Nakanishi, Turner and Burg found that betaine, like inositol, is accumulated by MDCK cells in hyperosmotic medium because of increased transport into the cells. There is a large increase in  $V_{max}$ . The mechanisms involved may be the same as for inositol and the investigators are proceeding with studies along similar lines.

Nakanishi and Burg found that accumulation of GPC by MDCK cells in hyperosmotic medium is due to synthesis from choline. In contrast to the systems for the other osmolytes, the trigger apparently is osmolality, per se, rather than intracellular ionic strength. Accumulation depends on choline in the medium.

Berkowitz, Balaban, Nakanishi, and Burg are tracing the metabolic pathway by placing  $^{13}\text{C}$ -choline in the medium and using NMR to determine which compounds the  $^{13}\text{C}$  passes through. Once the rate limiting step is identified, they will attempt to isolate the corresponding enzyme and proceed as for aldose reductase.

Yancey and Burg studied the effect of osmolyte accumulation on cell viability, using cloning efficiency as a measure of cell survival and growth, as follows:-

1.) Yancey previously noted that urea and trimethylamines generally appear together as organic osmolytes. He showed that either urea or a trimethylamine alone perturbs enzyme function in vitro, but that this presumably harmful effect is neutralized when both are present in the ratio (2:1) found in marine animals. Both urea and trimethylamines (betaine and GPC) are present in renal medullas. Yancey and Burg tested their effects separately and together on the cloning efficiency of renal cells (MDCK). Either urea or betaine alone depressed cloning efficiency, but combined they enhanced it, confirming Yancey's theory and providing a rationale for the occurrence of methylamines in the renal medulla where urea concentration is high as part of the concentrating mechanism.

2.) Sorbitol is the principal organic osmolyte that appears in GRB-PAP1 cells in hyperosmotic medium. Bagnasco and Burg found that when its accumulation was prevented by aldose reductase inhibitors, the cloning efficiency decreased in hyperosmotic media, but not in media with normal osmolality.

3.) Some of the complications of diabetes are believed to occur because hyperglycemia causes abnormal accumulation of sorbitol in cells (e.g. in ocular lens and peripheral nerves) that contain aldose reductase, and the complications are prevented by inhibitors of aldose reductase. In order to test whether this theory also applies to renal cells, Yancey and Burg elevated glucose in the medium bathing GRB-PAP1. Large amounts of sorbitol accumulated. This reduced cloning efficiency significantly, and the effect was prevented by aldose reductase inhibitors, which prevented the accumulation of glucose by the cells. In a control cell line (CHO) that does not express aldose reductase, neither high glucose or the inhibitors had any significant effect.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01237-10 KE

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Hormonal control of transport in kidney epithelia in culture

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	Joseph S. Handler	Section Chief	LKEM, NHLBI
Others	Agnes S. Preston	Chemist	LKEM, NHLBI
	Chester Williams	Biologist	LKEM, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

Membrane Metabolism Section

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, MD 20982

TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER:

2.5

2.0

0.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The transport effects and the mechanism of action of hormones and other factors are studied in epithelia formed in culture. To understand the responses better, the cell biology of the epithelia is studied as well as events specific to transport. Techniques of somatic cell genetics are applied to manipulate the sodium-coupled phosphate transporter.

567



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01266-06 KE

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Control of epithelial cell volume

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Kenneth R. Spring	Res. Physiologist	LKEM, NHLBI
Others:	Jeffrey L. Garvin	Guest Worker	LKEM, NHLBI
	R. Joel Lowy	Senior Staff Fellow	LKEM, NHLBI
	Arthur Siebens	Senior Staff Fellow	LKEM, NHLBI
	Timothy J. Furlong	Visiting Associate	LKEM, NHLBI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, MD 20892

## TOTAL MAN-YEARS:

5.0

## PROFESSIONAL:

5.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Large quantities of salt and water move across epithelial cells. These cells are able to maintain a constant volume by balancing solute entry and exit. The mechanisms for epithelial cell volume regulation are under investigation in this laboratory. Optical and microelectrode studies have been performed on the gallbladder of Necturus, on the renal cortical collecting tubule of the rabbit, the toad skin, on cultured renal papillary epithelium.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 HL 01282-02 KE
PERIOD COVERED <p style="text-align: center;">October 1, 1987 to September 30, 1988</p>		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <p style="text-align: center;">Solute and Water Transport in Renal Epithelia</p>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <div style="display: flex; justify-content: space-between;"> <div style="width: 60%;">           P.I.: Mark A. Knepper            Others:              J.M. Sands              J.L. Garvin              H. Nonoguchi/ Y. Terada              S.M. Wall              C.-L. Chou              Randall Packer         </div> <div style="width: 35%; text-align: right;">           Senior Investigator             Senior Staff Fellow            Guest Worker            Visiting Fellows            Senior Staff Fellow            Guest Worker            Guest Worker         </div> </div>		
COOPERATING UNITS (if any) <div style="display: flex; justify-content: space-between;"> <div style="width: 60%;">           Raymond Mejia            M.B. Burg         </div> <div style="width: 35%; text-align: right;">           Mathematician            Chief         </div> </div>		
LAB/BRANCH <p style="text-align: center;">Laboratory of Kidney and Electrolyte Metabolism</p>		
SECTION		
INSTITUTE AND LOCATION <p style="text-align: center;">National Heart, Lung and Blood Institute, NIH, Bethesda MD 20892</p>		
TOTAL MAN-YEARS: <p style="text-align: center;">5.55</p>	PROFESSIONAL:	OTHER:
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <div style="width: 30%;"> <input type="checkbox"/> (a) Human subjects  <input type="checkbox"/> (a1) Minors  <input type="checkbox"/> (a2) Interviews         </div> <div style="width: 30%;"> <input type="checkbox"/> (b) Human tissues         </div> <div style="width: 35%;"> <input checked="" type="checkbox"/> (c) Neither         </div> </div>		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>           The kidney contains several distinct epithelia that, in their aggregate function, are responsible for formation of the urine. We are studying the roles of these epithelia in the regulation of the excretion of water, urea, ammonium, bicarbonate, sodium, potassium, and chloride. The general approach is to dissect the epithelia from the kidney and to study their functions in vitro. Experiments in the cortical collecting duct of rat showed that atrial natriuretic factor (ANF) directly inhibits NaCl and fluid absorption. Experiments in rat inner medullary collecting ducts revealed that ANF markedly inhibits vasopressin-stimulated osmotic water permeability by a "post-cyclic AMP" effect. Mathematical modelling studies have demonstrated that ANF effects in the collecting duct system are quantitatively sufficient to account for increases in renal NaCl and fluid excretion. Isolated perfused tubule studies have demonstrated that vasopressin-stimulated urea transport in the rat inner medullary collecting duct is saturable, is inhibited by chemical analogs of urea, is inhibited by phloretin, and is independent of the vasopressin-stimulated water permeability pathway. These results support the view that the urea transport occurs via a specialized urea carrier or channel. Experiments in terminal inner medullary collecting ducts have demonstrated luminal acidification, which is increased by in vivo acidosis or deoxycorticosterone administration and by vasopressin in vivo. Experiments in microdissected rat collecting ducts have demonstrated that the terminal inner medullary collecting duct possesses substantial Na-K-ATPase activity which is enhanced by dietary NaCl restriction or mineralocorticoid administration. Experiments in isolated rabbit papillary surface epithelium have demonstrated that vasopressin reduces passive chloride permeability.         </p>		



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01283-01 KE

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Role of organic osmolytes in renal cells.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	Maurice Burg	Chief	LKEM, NHLBI
Other:	Toshiki Moriyama	Visiting Fellow	LKEM, NHLBI
	Arlyn Garcia-Perez	Staff Fellow	LKEM, NHLBI
	Helen Murphy	Chemist	LKEM, NHLBI
	Benjamin Cowley	Guest Worker	LKEM, NHLBI
	Takeshi Nakanishi	Visiting Fellow	LKEM, NHLBI
	Paul Yancey	I. P. A.	LKEM, NHLBI
	Moo Kwon	Visiting Fellow	LKEM, NHLBI

## COOPERATING UNITS (if any)

Joseph Handler	Section Chief	LKEM, NHLBI
----------------	---------------	-------------

## LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, MD 20205

## TOTAL MAN-YEARS:

8.3

## PROFESSIONAL:

8.3

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The osmolality of the blood in the renal inner medulla is high and varies with the urinary concentration. Both NaCl and urea are elevated. The medullary cells evidently survive and function in this adverse environment. The present studies are concerned with understanding the mechanisms involved. When cells are stressed by a high salt environment, they generally accumulate osmotically active organic solutes ("osmolytes") in order to maintain a favorable internal milieu, while regulating their volume. We identified the organic osmolytes in renal inner medullary cells as glycerophosphorylcholine (GPC), betaine, sorbitol, and inositol, and showed that the osmolyte levels varied with urine concentration (and, presumably, medullary salt concentration). We are now using renal cell cultures to study the mechanism and control of osmoregulatory accumulation of these organic osmolytes.

578





Annual Report  
Laboratory of Molecular Cardiology  
National Heart, Lung, and Blood Institute  
October 1, 1987 through September 30, 1988

The Laboratory of Molecular Cardiology is carrying out studies on the regulation and expression of vertebrate contractile proteins. Our studies are focused on understanding the role and mechanism of regulation of smooth muscle and cytoplasmic myosin, as well as related contractile proteins, in a variety of cells. Myosin is a protein with a molecular weight of 460,000, and in vertebrate cells is usually composed of six polypeptide chains, two with a molecular weight of 200,000 (myosin heavy chains) and two pairs of light chain with molecular weights of approximately 20,000 and 17,000. The regulation of contractile activity in vertebrate smooth muscle and nonmuscle cells, such as intestinal brush border cells, platelets and basophils differs from that found for cardiac and skeletal muscle cells. In cardiac and skeletal muscle cells, contractile activity is initiated when calcium binds to troponin, which is located on the actin filaments. In the case of vertebrate smooth muscle and nonmuscle cells, regulation of the actin-myosin interaction involves the myosin molecule more directly. The initiation of contractile activity in these cells follows a rise in intracellular calcium concentration, but instead of binding to the troponin C subunit of the troponin complex, calcium binds to calmodulin. The calcium-calmodulin complex activates the enzyme myosin light chain kinase, which in turn results in phosphorylation of myosin at a specific serine residue on the 20,000 dalton light chain of myosin.

Phosphorylation appears to have at least two important effects on cytoplasmic and smooth muscle myosin. It increases the actin-activated MgATPase activity, which is necessary for the cycling of the actin-myosin crossbridges, and it enhances the ability of myosin molecules to form filaments. These effects may well be interdependent. Based on biochemical, physiological and pharmacological evidence, it appears that phosphorylation of smooth muscle and cytoplasmic myosin is necessary for the onset of contractile activity. However, phosphorylation may not be necessary for the maintenance of smooth muscle tension and the equivalent biological process in nonmuscle cells. A number of possible mechanisms have been postulated for the maintenance of smooth muscle tension, following the initiation of contractile activity, including a role for other proteins, such as caldesmon, and even a low level myosin phosphorylation by itself.

Our laboratory has continued to study the role of myosin light chain phosphorylation in regulating contractile activity in smooth muscle and nonmuscle cells. We are also studying the occurrence and function of myosin heavy chain phosphorylation in both smooth muscle and nonmuscle cells. In addition, we have made significant progress in cloning the cDNA for cytoplasmic myosin and have initiated the cloning of the cDNA for the regulatory protein, caldesmon from chicken intestinal brush border. We have also begun work on cloning the gene for cytoplasmic myosin.



Growth and Differentiation of Smooth Muscle and Nonmuscle Cells (S. Kawamoto Z01 HL 01665-13 MC) During the past year we described the occurrence of myosin heavy chain phosphorylation in smooth muscle cells found in intact rat aorta and in cultured aorta cells. The extent of heavy chain phosphorylation was quantitated in cultured cells and found to be approximately 0.7 mole phosphate/mole myosin heavy chain. Working with human platelets, we also found that treatment with phorbol esters results in significant amounts of both myosin heavy chain and light chain phosphorylation. Specific serine residues on both the myosin heavy chain and light chain are phosphorylated and these sites appear to be the same sites phosphorylated by protein kinase C, in vitro.

Myosin and Caldesmon Phosphorylation in Nonmuscle Cells (J.M. Hettasch, J.R. Sellers Z01 HL 01785-09 MC) The purpose of this project is to examine the physiological role of caldesmon in intact human platelets. In vitro caldesmon, which binds both actin and calmodulin, has been shown to inhibit the actin-activated MgATPase activity of myosin. This inhibition is relieved in the presence of calcium and calmodulin. Human platelets, which have been equilibrated with  $^{32}\text{P}$  have been treated with phorbol esters to see if this treatment will result in phosphorylation of caldesmon. The stoichiometry and sites of phosphorylation will be monitored and the results will be compared to in vitro studies, which will utilize a number of different kinases. During the past year we found that platelet caldesmon can be phosphorylated by protein kinase C (1 mole phosphate/mole caldesmon) and also by  $\text{Ca}^{2+}$ -CaM kinase II. The significance of this phosphorylation both in vitro and in vivo, is presently under study.

Role of Phosphorylation as a Regulatory Mechanism in Muscle Contraction (S. Umemoto, J.R. Sellers Z01 HL 01786-09 MC) The Nitella-based in vitro motility assay has been used to study the movement of beads coated with various myosins on cables of Nitella actin. Using this assay, it was found that phosphorylation by the enzyme myosin light chain kinase at serine-19 on the 20,000 Da light chain of myosin isolated from turkey gizzard, bovine trachea, bovine aorta and human platelets was required for bead movement. This is in contrast to the results with beads coated with skeletal muscle myosin. Phosphorylation of skeletal muscle myosin had no effect on bead movement, which was relatively rapid (2 u/s) compared to beads coated with phosphorylated gizzard (0.2 u/s) and platelet myosin (0.04 u/s). Phosphorylation on threonine-18 by myosin light chain kinase (in addition to serine-19) had no effect on the velocity of movement of beads coated with smooth muscle myosin. Phosphorylation of the 20,000 Da myosin light chain by protein kinase C also had no effect on bead movement.

Phosphorylation as a Regulatory Mechanism (M.A. Corson Z01 HL 04202-07 MC) The purpose of this project is to use a number of different agonists, particularly those that have not been well-studied, to contract strips of smooth muscle from bovine trachea. The peptide Substance P is a prime example of this type of agonist and we have determined that the contractile response seen following treatment with this agent is associated with an increase in myosin light chain phosphorylation. The



sites of phosphorylation are being characterized and the stoichiometry of phosphorylation is being measured.

Structure, Function and Expression of Myosin and Myosin Light Chain Kinase (M.A. Conti, Z01 HL 04205-06 MC) We are carrying on experiments to study the effect of phosphorylating the heavy chain of avian brush border myosin, as well as other cytoplasmic myosins, such as that found in platelets. These experiments are being carried out in conjunction with cloning and sequencing the cDNA of vertebrate cytoplasmic myosin (see Z01 HL 04208-02 MC) By combining biochemical studies on the structure and function of cytoplasmic myosins with site-directed mutagenesis of myosin heavy chain cDNA we hope to learn which residues of the myosin heavy chain are important for function and what effects heavy chain phosphorylation have on myosin function, both in vitro and in situ.

Regulation of Genes for Contractile Proteins in Muscle and Nonmuscle Cells (L. Weir, M. Simons Z01 HL 04207-03 MC) In an effort to identify cis and trans acting factors that regulate the genes for the contractile proteins in nonmuscle cells, we have isolated a 17 kb clone from a human genomic library which contains part of the gene for the heavy chain of nonmuscle myosin. Present plans are to use 5' and 3' parts of the 17kb clone to complete the isolation of the entire gene. We suspect that the gene may be alternatively spliced in different tissues and we will study this using nuclease protection assays.

Cloning of the cDNA for Nonmuscle Myosin Heavy Chain (R.V. Shohet, D.A. Brill, M.A. Conti, S. Kawamoto Z01 HL 04208-02 MC) We have identified cDNA clones for chicken brush border myosin heavy chain that encode over 90% of the amino acid sequence. We have compared our avian clones with human clones isolated from a lymphocyte library. Our immediate plans are to complete the nucleotide sequences of both the coding and untranslated regions, to compare the brush border nonmuscle clones with those obtained from other tissues and species and to express the brush border cytoplasmic myosin heavy chain clone in E. Coli. (See also Z01 HL 04205-06 MC)

Myosin Phosphorylation and Basophil Secretion (I. Peleg Z01 04209-02 MC; in cooperation with R. Ludowycke and M. Beaven, NHLBI, CP) When rat leukemic basophils are activated by aggregation of their receptors for IgE, there is a marked increase in phosphorylation of the basophil myosin. We have identified the sites of phosphorylation as serine 1 and/or 2 on the 20,000 Da myosin light chain and an apparently unique serine site on the 200,000 Da myosin heavy chain. The extent of phosphorylation is significant in that prior to activation, the sites on the heavy chain and light chain appear to be devoid of phosphate, but after activation, there is approximately 0.5-0.8 moles of phosphate/mole of heavy chain and light chain. The enzyme responsible for this phosphorylation appears to be protein kinase C. Since the onset of histamine secretion is preceded by this phosphorylation, we are presently investigating a possible causal relationship between basophil myosin phosphorylation and histamine secretion.

Role of Heavy Chain Phosphorylation in Contractile Activity (C.A. Kelley Z01 HL 04210-01) We have recently identified two kinases that



can catalyze the phosphorylation of the myosin heavy chain isolated from bovine aorta smooth muscle cells. Both casein kinase II and calmodulin-dependent kinase II can incorporate approximately 0.5 moles of phosphate/mole of myosin heavy chain. Tryptic peptide maps of the phosphorylated heavy chains revealed 1 major and 2 to 3 minor peptides in both cases. The major phosphorylated peptide in each case appears to be the same, based on its migration in tryptic peptide maps. We are presently studying the effects of myosin heavy chain phosphorylation on the biological activity of myosin using a number of in vitro assays.

Cloning of the cDNA for Avian Intestinal Epithelial Brush Border Caldesmon (J. Sellers, M.A. Conti Z01 HL 04211-01 MC) Using an affinity purified anti-caldesmon antibody, a cDNA library constructed from chicken intestinal epithelial brush border cells was screened. (The library was provided by P. Matsudaira of MIT.) Two clones have been characterized to date, with inserts of approximately 2 kb and 3 kb. Each clone has an internal EcoRI restriction site. These clones are presently being characterized with respect to Northern blot analysis, restriction sites and nucleotide sequences. The 2.1 kb insert recognizes mRNA with sizes of 5 and 3 kb, isolated from chicken intestinal brush border and kidney. These messages presumably correspond to the heavy and light forms of caldesmon. The ultimate goal of these studies is to understand the mechanism by which caldesmon might modulate contractile activity. Characterization and expression of these cDNA clones will enable us to initiate structure-function studies on caldesmons which has been altered at specific amino acid residues, including the sites of phosphorylation.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

## PERIOD COVERED

October 1, 1987 through September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Growth and Differentiation of Smooth Muscle and Nonmuscle Cells

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Sachiyo Kawamoto, M.D., Ph.D., Visiting Associate, LMC, NHLBI  
Robert S. Adelstein, M.D., Chief, LMC, NHLBI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Molecular Cardiology

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

0.9

## PROFESSIONAL:

0.9

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Reversible phosphorylation of proteins is thought to be one mechanism for regulation of various cell functions. We have been studying phosphorylation of myosin heavy chains (MHCs) and myosin light chains (MLCs), using the primary culture cells derived from rat aorta, as well as human platelets purified from platelet-rich plasma. We found that both MHC and MLC can be phosphorylated by protein kinase C at significant levels when human platelets were treated with the tumor promoting phorbol ester, 12-O-tetradecanoylphorbol 13-acetate (TPA). The site phosphorylated on the platelet MHC was localized to serine residue(s) in a single major tryptic peptide. The stoichiometry of this phosphorylation was approximately 0.7 moles of phosphate/mole of MHC. The phosphorylation of MLCs was also observed at serine residues in TPA-treated platelets. The phosphorylated sites in platelet MLCs corresponded to Serine 1 and Serine 2 in MLCs from turkey gizzard and the stoichiometry of MLC phosphorylation was approximately 1.0 mole of phosphate/mole of MLC. The physiological meaning of this phosphorylation is under study.

SP7



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01785-09 MC

## PERIOD COVERED

October 1, 1987 through September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Myosin and Caldesmon Phosphorylation in Nonmuscle Cells

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Joanne M. Hettasch, Ph.D. Staff Fellow, LMC, NHLBI  
James R. Sellers, Ph.D. Research Biologist, LMC, NHLBI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Molecular Cardiology

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.3

## PROFESSIONAL:

1.3

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Caldesmon is a calmodulin and actin binding protein which has been shown to decrease actin-activated myosin MgATPase activity. This inhibition can be reversed in the presence of calcium and calmodulin. It has been suggested that caldesmon may play a role in the regulation of actin-myosin interaction.

Although the concentration of calcium may influence the ability of caldesmon to exert its effect, other evidence suggests that another regulatory mechanism may also be important. It has been shown that the inhibitory effect of caldesmon on actin-activated myosin MgATPase can be reduced following phosphorylation of caldesmon by a CaM-dependent protein kinase. Caldesmon also appears to be a substrate for protein kinase C. More recently, it was demonstrated that protein kinase C phosphorylates caldesmon when intact platelets are stimulated with phorbol esters. These observations suggest that phosphorylation of caldesmon may play a role in the cytoskeletal and/or contractile changes which occur in the platelet during activation. This project will examine the physiologic role of caldesmon in the intact platelet.

590



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01786-09 MC

PERIOD COVERED

October 1, 1987 through September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Role of Phosphorylation as a Regulatory Mechanism in Muscle Contraction

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Seiji Umemoto, M.D., Ph.D., Visiting Fellow, LMC, NHLBI  
James R. Sellers, Ph.D., Research Biologist, LMC, NHLBI  
Estelle V. Harvey, Biologist, LMC, NHLBI  
William A. Anderson, Jr., Chemist, LMC, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Cardiology

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

2.5

PROFESSIONAL:

1.3

OTHER:

1.2

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The mechanism of the phosphorylation-dependent myosin-linked regulation of smooth muscle and nonmuscle myosins is being investigated. We compared the the conventional steady-state kinetic analysis of the actin-activated myosin MgATPase activity with the Nitella-based in vitro motility assay which is a quantitative assay for measuring the velocity of myosin-coated beads over an organized substratum of actin, and analyzed the effect of phosphorylation of various sites on the 20,000 Da light chain of smooth muscle and cytoplasmic myosins. We also determined the effect of tropomyosin on the actin-activated myosin MgATPase activity and on the movement of myosin-coated beads.

593



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 HL 04202-07 MC

PERIOD COVERED

October 1, 1987 through September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Phosphorylation as a Regulatory Mechanism

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Marshall A. Corson, M.D., Medical Staff Fellow, LMC, NHLBI

Robert S. Adelstein, M.D., Chief, LMC, NHLBI

William A. Anderson, Jr., Chemist, LMC, NHLBI

COOPERATING UNITS (if any)

Mark Schoenberg, M.D., LPB, NIAMS

LAB/BRANCH

Laboratory of Molecular Cardiology,

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

1.5

PROFESSIONAL:

1.2

OTHER:

0.3

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A system for studying basic biochemical and physiologic aspects of the contraction of intact mammalian smooth muscle has been further developed during the past year. Utilizing fresh strips of bovine trachealis muscle, methods have been refined to correlate the contractile response to certain agonists with the state of phosphorylation of myosin light and heavy chains, and myosin light chain kinase (MLCK). To determine whether force generation can be catalyzed by phosphorylation of myosin light chain (MLC) at amino acid residues other than the preferential MLCK site (serine 19), a phorbol ester activator of intracellular protein kinase C (PKC) and the physiologic peptide agonist Substance P have been used to cause contraction. In both cases the pattern of MLC phosphorylation qualitatively resembles that seen with other conventional methods of activation, with some variation in quantitative aspects. In addition, experiments have been undertaken to characterize biochemical correlates of tracheal relaxation, with particular interest in the response due to substances acting through cyclic AMP.

596





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 HL 04205-06 MC

PERIOD COVERED

October 1, 1987 through September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structure, Function and Expression of Myosin and Myosin Light Chain Kinase

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Mary Anne Conti, Ph.D., Research Chemist, LMC, NHLBI  
Robert S. Adelstein, M.D., Chief, Laboratory of Molecular Cardiology, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Cardiology

SECTION

INSTITUTE AND LOCATION

Nation Heart, Lung, and Blood Institute, NIH, Bethesda, MD, 20892

TOTAL MAN-YEARS:

0.4

PROFESSIONAL:

0.4

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☒ (b) Human tissues    ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We are investigating the site(s) of phosphorylation of the nonmuscle myosin heavy chain and the effect of phosphorylation on myosin functions, such as MgATPase activity and filament formation.

599



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 04207-03 MC

PERIOD COVERED

October 1, 1987 through September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Genes for Contractile Proteins in Muscle and Nonmuscle Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Lawrence Weir, Ph.D., Visiting Associate, LMC, NHLBI  
Michael Simons, M.D., Medical Staff Fellow, LMC, NHLBI  
Robert S. Adelstein, M.D., Chief, LMC, NHLBI

COOPERATING UNITS (# any)

LAB/BRANCH

Laboratory of Molecular Cardiology

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

2.1

PROFESSIONAL:

2.1

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have isolated a clone from a human genomic library which contains part of the gene for the heavy chain of nonmuscle myosin. This clone has been analyzed by restriction mapping, Southern blotting and DNA sequencing. In this way, we are elucidating the structure of the gene, the knowledge of which is essential to studies of how the gene is regulated.

601



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 04208-02 MC

PERIOD COVERED

October 1, 1987 through September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cloning of the cDNA for Nonmuscle Myosin Heavy Chain

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Ralph V. Shohet, M.D., Medical Staff Fellow, LMC, NHLBI  
David A. Brill, M.D., Medical Staff Fellow, LMC, NHLBI  
Mary Anne Conti, Ph.D., Research Chemist, LMC, NHLBI  
Sachiyo Kawamoto, M.D., Ph.D., Visiting Associate  
Robert S. Adelstein, M.D., Chief, LMC, NHLBI  
Yvette Preston, Biologist, LMC, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Cardiology

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

4.0

PROFESSIONAL:

3.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Using affinity purified polyclonal antibodies a 3.6kb cDNA clone of chicken nonmuscle myosin heavy chain (MHC) was isolated from a lambda gt11 library of intestinal brush border. A 5' fragment of this clone was used to obtain further clones from a human lymphocyte cDNA library, which in turn, has helped to extend the chicken brush border sequence towards the 5' terminus. We have thus been able to compare these sequences of vertebrate nonmuscle MHC between species. To obtain complete sequence data on a nonmuscle MHC, we have produced new libraries, using random primers and specific oligonucleotides from our established sequence. We have also begun biochemical studies of the cloned fragments, expressing the light meromyosin encoded by our initial clone, in E. coli.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 04209-02 MC

PERIOD COVERED

October 1, 1987 through September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Myosin Phosphorylation and Basophil Secretion

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Itzhak Peleg, Ph.D., Visiting Fellow, LMC, NHLBI  
Robert S. Adelstein, M.D., Chief, LMC, NHLBI

COOPERATING UNITS (if any)

Russell Ludowyke, Ph.D., CP, NHLBI  
Michael Beaven, Ph.D. CP, NHLBI

LAB/BRANCH

Laboratory of Molecular Cardiology

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

1.2

PROFESSIONAL:

1.2

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The role of myosin phosphorylation in histamine secretion from rat basophil leukemic (RBL) cells has been investigated. In unstimulated cells histamine secretion was about 1% of the total cell content and the stoichiometry of myosin phosphorylation was 1 mol phosphate/mol heavy chain and 0.4 mol phosphate/mol light chain. In the case of the myosin light chains, all of the phosphate was confined to a single site known to be phosphorylated by myosin light chain kinase. Upon stimulation for 10 min with DNP-BSA, the amount of histamine secretion from the cells reached 40-50% of the total content and the stoichiometry of phosphorylation was 1.78 mol phosphate/mol heavy chain and 0.84 mol phosphate/mol light chains. In this case, two distinct populations of light chains were detected: monophosphorylated and diphosphorylated, each of which contained a mixture of sites phosphorylated by myosin light chain kinase and protein kinase C. We are currently investigating the effect of specific inhibitors/activators of these two kinases in order to establish the specific role of myosin phosphorylation in histamine secretion from these cells.

605





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 04210-01 MC

## PERIOD COVERED

October 1, 1987 through September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Role of Heavy Chain Phosphorylation in Regulation Contractile Activity

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Christine A. Kelley, Ph.D., Staff Fellow, LMC, NHLBI

Robert S. Adelstein, M.D., Chief, LMC, NHLBI

William A. Anderson, Jr., Chemist, LMC NHLBI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Molecular Cardiology

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.4

## PROFESSIONAL:

1.1

## OTHER:

0.3

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Vertebrate smooth muscle myosin heavy chain phosphorylation was recently observed in cultured aortic smooth muscle cells as well as in strips of intact aorta by our laboratory (Kawamoto and Adelstein, J. Biol. Chem. 263:1099-1102, 1988). We are now characterizing heavy chain phosphorylation in vitro using purified aortic smooth muscle myosin and purified kinases. We have identified two kinases that phosphorylate the myosin heavy chain. The stoichiometry of phosphorylation by these kinases has been determined, and the sites phosphorylated by the two kinases have been compared by two-dimensional tryptic peptide mapping of the phosphorylated heavy chains. We are currently interested in determining the exact sites of phosphorylation and the effects of heavy chain phosphorylation on myosin filament assembly and MgATPase activity.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 HL 04211-01 MC

PERIOD COVERED

October 1, 1987 through September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cloning of the cDNA for Avian Intestinal Epithelial Brush Border Caldesmon

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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Mary Anne Conti, Ph.D., Research Chemist, LMC, NHLBI  
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COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Cardiology

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

0.7

PROFESSIONAL:

0.5

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Caldesmon is a thin filament calmodulin-binding protein which appears to play a role in the regulation of actin-myosin interaction in smooth muscle and nonmuscle cells. There are at least 2 isoforms of caldesmon, a high molecular weight form found primarily in smooth muscle and a lower molecular weight form found mainly in nonmuscle cells. We are attempting to clone and sequence the caldesmon found in chicken intestinal brush border. Once this is accomplished we would like to use bacterial expression vectors and study the expressed protein as well as genetically engineered variants.

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Molecular Disease Branch  
National Heart, Lung, and Blood Institute  
October 1, 1987 through September 30, 1988

The overall objective of the research program of the Molecular Disease Branch is the elucidation of the molecular and structural properties of the human plasma apolipoproteins (apo), the physiological role of the apolipoproteins and lipoproteins in lipid transport, the determination of the mechanisms involved in the regulation of cellular cholesterol metabolism and transport, the elucidation of the metabolic and molecular mechanisms involved in plasma lipoprotein synthesis, transport, and catabolism in normal individuals and patients with disorders of lipid metabolism and atherosclerosis, and the elucidation of the mechanisms involved in the development of atherosclerosis.

During the last several years the staff of the Molecular Disease Branch has developed a conceptual framework for the understanding of the dynamic processes involved in the biosynthesis, transport, and catabolism of plasma apolipoproteins and lipoproteins. Within this framework the plasma lipoproteins are conceptualized as a polydisperse collection of lipoproteins, the apolipoprotein composition of which is determined by the laws of mass action. The constituent of the lipoprotein particle which is responsible for the regulation of the lipoprotein particle transport and metabolism is the apolipoprotein moiety. The distribution of a specific apolipoprotein within plasma is governed by the relative concentration of and affinity for the individual plasma lipoproteins. This concept of plasma lipoproteins emphasizes the fundamental importance of the apolipoprotein in regulating metabolism and provides a framework for understanding apolipoprotein-lipoprotein interactions during lipoprotein biosynthesis, transport, and catabolism in normal man and in patients with dyslipoproteinemias and atherosclerosis.

The determination of specific physiological functions of the individual plasma apolipoproteins continues to be of major importance in our understanding of the role of apolipoproteins in lipoprotein structure, function, and metabolism. Based on our current information, apolipoproteins have been shown to be of importance in four general areas of lipoprotein metabolism: 1) cofactor for enzymes (apoC-II for lipoprotein lipase, apoA-I for lecithin cholesterol acyltransferase); 2) ligand on the lipoprotein particle for interaction with high affinity receptor sites (apoB-100 on LDL, apoA-I on HDL; 3) exchange proteins (e.g. apoD) for phospholipids, cholesteryl esters, and triglycerides; and 4) structural component for the lipoprotein particle (apoA-I for HDL, apoB-100 for chylomicrons as well as LDL, and apoB-48 for the chylomicrons).

Prerequisite to our understanding of the physiological and biochemical role of apolipoproteins in lipid and lipoprotein metabolism is a detailed knowledge of the molecular structure and function of the plasma apolipoproteins. Over the last several years we have systematically evaluated the molecular structure and primary amino acid sequence of apolipoproteins A-I, A-II, apoC-I, apoC-II, apoC-III, and apoB-100.



Over the last three years a major focus of research in the Branch has been apoB. ApoB is present in plasma as two isoforms, designated apoB-100 and apoB-48, with a molecular weight of 510,000 and 250,000 daltons respectively. ApoB-100 is the major apolipoprotein in LDL, and interacts with the LDL receptor initiating LDL endocytosis and catabolism. In our initial studies the complete cDNA and derived 4536 amino acid sequence of apoB-100 was determined. ApoB-100 contains a cluster of 15 cysteine residues near the amino terminus of apoB-100, and 20 potential N-linked glycosylation sites.

Of particular interest has been the elucidation of the mechanism for the biosynthesis of apoB-48. A single gene for apoB is located on chromosome 2. Studies utilizing apoB monoclonal antibodies indicated that apoB-48 was located at the amino terminal half of apoB-100. To further define the structural relationship between apoB-100 and apoB-48, cDNA probes were selected which were localized to the middle region of apoB-100 cDNA, and used to screen human intestinal cDNA libraries. Clones which hybridized to the cDNA probes were subcloned into M13, and the nucleotide sequences determined by the dideoxy method. Clones were obtained which contained the 'CAA' codon coding for glutamine at nucleotides 6666-6669 in apoB-100, and 'TAA' a premature stop codon. The C → T substitution was at nucleotide 6666. These studies provided for the first time a mechanism for the biosynthesis of apoB-48. The change in the 'CAA' codon for glutamine for the 'TAA' stop codon resulted in the synthesis of an apolipoprotein, apoB-48, which terminated at amino acid 2152. Therefore apoB-100 contains 4536 amino acids, and apoB-48 contains 2152 amino acids.

To determine if this C → T substitution was also present in genomic DNA, sequence specific oligonucleotide probes were synthesized which were specific for the glutamine and stop codons, thereby permitting the screening of genomic DNA to establish if the 'TAA' codon was present at the DNA level. Detailed Southern blot analysis as well as dideoxy sequencing of genomic clones revealed that only the 'CAA' codon was present in genomic DNA. The combined results from these studies definitively established that the mechanism for the production of apoB-48 is a unique RNA editing mechanism which involves the substitution of a premature stop codon for a glutamine codon. This RNA editing mechanism has not been previously described.

Following the elucidation of the RNA editing mechanism for the biosynthesis of apoB-48, studies were undertaken to determine if this mechanism was present in both the human liver as well as intestine. The sequence specific synthetic oligonucleotide probes were used for Northern blot analysis of liver and intestine mRNA, as well as to screen libraries which contained apoB sequences. Northern blot analysis of human liver and intestinal RNAs indicated that mRNAs containing both the 'CAA' and 'UAA' were present in both liver and intestine. To definitively establish that both the Gln and stop codons were present in both tissues, libraries were screened by Southern hybridization employing the two synthetic oligonucleotide probes which are specific for apoB-100 and apoB-48. Sequence analysis identified clones containing both the 'CAA' codon, as well as the 'TAA' stop codon. The data from these studies established that the RNA editing mechanism was





present in both liver and intestinal tissue. The finding that the intestine has the potential to secrete apoB-100 was of particular importance since apoB-100 containing remnant lipoproteins have been shown to be atherogenic.

The human liver was shown to contain mRNA having the 'UAA' stop codon, however, the percentage of stop codon and apoB-48 secretion is small. In contrast, the rat liver has been shown to secrete both apoB isoproteins, designated apoB<sub>L</sub> and apoB<sub>S</sub>. ApoB<sub>S</sub>, or small apoB, secretion is 60-70% of the total apoB<sub>S</sub>. To analyze the apoB isoproteins in rat liver, a rat liver cDNA library was screened using a rabbit cDNA probe which was complementary to the nucleotide sequence of the apoB glutamine codon. Clones which were positive to this probe were subcloned into M13, and the nucleotide sequences determined. The percentage of glutamine and stop codons present in the rat liver were determined by random selection of clones, and sequence analysis. Using these techniques, the glutamine as well as the premature stop codon - 'TAA' were identified in rat liver cDNA clones. Therefore the mechanism for the biosynthesis of apoB<sub>S</sub> was an RNA editing mechanism as was observed in human tissue in which the 'CAA' glutamine codon is replaced by a 'TAA' stop codon. Therefore the apoB<sub>L</sub> and apoB<sub>S</sub> isoproteins in the rat are equivalent to the apoB-100 and apoB-48 of the human. The percentage of the apoB mRNA message which contained the 'UAA' premature stop codon was approximately 85%, consistent with predominate apoB<sub>S</sub> or apoB-48 secretion from the rat liver.

Regulation of apolipoprotein gene expression continues to be a major focus of research in the Branch. Detailed studies have been conducted on apoA-I, the major apolipoprotein of the high density lipoproteins (HDL). The 5' regulatory region of apoA-I has been analysed to determine the locations of the regulatory elements and tissue specific regions of apoA-I. A 2.5 kbp genomic apoA-I clone was isolated from a genomic library, and the nucleotide sequence determined. A TATA box, CAT box like, and two GC sequences were identified 30, 108, 220, and 440 bp upstream respectively from the transcriptional start site in the intestine. Analysis of deletion mutants of the human apoA-I 5' flanking region revealed positive elements -250 to -199 bp, -487 to -413 bp, and -1021 and -691 upstream from the transcriptional start site. Elements for tissue specific repression of apoA-I gene expression are located -2067 to -1467 and -199 to -80 bp from the transcriptional start site.

The regulation of the apoB gene continues to be analyzed. The apoB gene regulation has been studied in normal hepatocytes and Hep G2 cells. The addition of LDL was associated with a reduction in apoB mRNA as well as a decrease in apoB in the media. These studies have been extended to include the correlation with HMG-CoA reductase activity which decreases with LDL incubation. The addition of 25-OH cholesterol to Hep G2 cells resulted in a decrease in HMG-CoA reductase activity, however, apoB in the media and apoB mRNA increased. These results have been interpreted as indicating that Hep G2 cells have separate metabolic pools of cholesterol and these pools have different effects on apolipoprotein gene expression.



The potential role of post-translational modifications of the plasma apolipoproteins in modulating apolipoprotein gene expression continues to be studied. ApoA-I has been shown to be phosphorylated by a calcium-calmodulin dependent protein kinase in vitro. ApoA-I was also phosphorylated in Hep G2 cells indicating that apoA-I could be phosphorylated both in vivo as well as in vitro. The site of phosphorylation of apoA-I was determined following incubation with the calcium-calmodulin dependent protein kinase. ApoA-I has shown by amino acid analysis to be phosphorylated at a serine residue. Isolation and automated Edman degradation of peptides of apoA-I generated by tryptic or cyanogen bromide cleave established the site of phosphorylation to be at residue 201, in the carboxyl terminal end of apoA-I. The identification of the single site of phosphorylation will now permit the analysis of the importance of phosphorylation on the metabolism of apoA-I by site directed mutagenesis. Phosphorylation as well as acylation may play a central role in biosynthesis, transport, and catabolism of the apolipoproteins.

Studies on the molecular defects in the human dyslipoproteins have been actively pursued during the last year. Initial studies were performed on abetalipoproteinemia, a disease characterized by malabsorption, ataxia, atypical retinitis pigmentosa, and hemolytic anemia. Patients with abetalipoproteinemia have low levels of plasma cholesterol and triglycerides, and virtually no plasma apoB and apoB containing lipoproteins. Analysis of the molecular defect in abetalipoproteinemia revealed that there were no major insertions or deletions in the apoB gene on chromosome 2. Hepatic apoB mRNA was elevated 5-6 fold and immunocytochemical studies with monoclonal apoB antibodies revealed that the B apolipoprotein was present in the hepatocyte. These studies indicated that the defect in abetalipoproteinemia was due to a defect in secretion of apoB from the hepatocyte.

Studies on the defect in homozygous hypobetalipoproteinemia have also been pursued. Homozygous hypobetalipoproteinemia patients have similar biochemical features as patients with abetalipoproteinemia except that oligate heterozygotes have a significant reduction in low density lipoproteins (LDL), and plasma apoB. In patients with homozygous hypobetalipoproteinemia there were no major insertions or deletions in the apoB gene, however, hepatic apoB mRNA and intracellular apoB were significantly reduced. The reduction in intracellular protein and mRNA, and the virtual absence of plasma apoB and LDL have been interpreted to indicate that the molecular defect in this disease is in the coding portion of the apoB gene with either failure of secretion of apoB or rapid catabolism of apoB after secretion into the plasma. To further elucidate the defect in this disease, the 5' flanking region of the apoB gene was cloned, and the sequence determined. There were two base substitutions upstream from the TATA box. To determine if these substitutions were of physiological significance a 800 base pair 5' flanking region of the apoB gene was analyzed utilizing the chloramphenicol acetyltransferase enzyme (CAT) assay. There was no significant difference in the CAT activity of constructs prepared from the 5' flanking region of the apoB gene from normal and homozygous hypobetalipoproteinemic subjects. These studies indicate that the 5'



flanking region of the apoB gene is normal in patients with homozygous hypobetalipoproteinemia, consistent with the conceptualization that the defect in this disease is a defect in the structural portion of the apoB gene.

Research continues in the Branch on the molecular defects in patients with apoC-II deficiency. ApoC-II deficient patients have severe hypertriglyceridemia, elevated plasma chylomicrons, eruptive xanthomas, and recurrent bouts of pancreatitis. Seven different kindreds are under investigation. The molecular defects in two kindreds have now been established by dideoxy sequencing of the cloned apoC-II gene. One kindred from Hamburg, Germany has a single base substitution (guanosine for a cytosine) at the first base of the donor splice site of intron II of the apoC-II gene. This mutation will abolish normal splicing of the apoC-II gene, and leads to reduced hepatic levels of apoC-II mRNA, and the absence of plasma apoC-II. The second proband from Nijmegen, the Netherlands was shown by sequence analysis to have a deletion of a cytosine at position 2943 of exon 3 of the apoC-II gene. This mutation results in the formation of a premature termination codon at a position corresponding to amino acid 18 of normal apoC-II. The mutation leads to the formation of a truncated 17 amino acid protein which is biologically inactive. A third kindred from Padova, Italy which is being analyzed is of particular interest since the proband has a apoC-II variant designated apoC-II<sub>Padova</sub>. This apoC-II variant is slightly lower in molecular weight and has a different pI than normal apoC-II. There were no major insertions or deletions in the apoC-II gene by Southern analysis. Hepatic apoC-II mRNA was approximately normal in concentration. Immunohistochemical studies revealed normal to increased amounts of intra-hepatic C-II apolipoprotein. The defect in this kindred from Padova with normal intrahepatic apoC-II mRNA and protein, but a deficiency of apoC-II in plasma appears to be a mutation in the coding portion of the apoC-II gene which results in defective hepatic secretion of the apoC-II variant, or an enhanced catabolism of secreted apoC-II leading to a virtual complete deficiency of plasma apoC-II.

The elucidation of the molecular defects in these kindreds with apoC-II deficiency has lead to the discovery of the heterogeneity in the molecular defect in this relatively rare genetic dyslipoproteinemia. Knowledge of the precise molecular defects in apoC-II deficiency will also permit more effective diagnosis of heterozygotes in kindreds with apoC-II deficiency. Studies are currently underway to elucidate the molecular defect in each of the other kindreds currently under study in the Branch.

The study of the coordinate regulation of apolipoprotein gene expression and cholesterol biosynthesis continues to be actively pursued in the Branch. The regulation of cholesterol biosynthesis has been actively investigated, and rat and human hepatic HMG-CoA reductases have been shown to be modulated in vivo and in vitro by a bicyclic cascade system involving reversible phosphorylation of both HMG-CoA reductase and reductase kinase. HMG-CoA<sub>2</sub> reductase is also regulated in vitro by both protein kinase C and a Ca<sup>2+</sup> calmodulin dependent kinase. These



studies have established that the activity of HMG-CoA reductase is modulated by several kinase systems.

The phosphorylation of apoA-I and apoB in vitro as well as during biosynthesis and secretion from Hep G2 cells, in conjunction with reversible phosphorylation of HMG-CoA reductase provide a mechanism for the coordinate regulation of cholesterol and apolipoprotein biosynthesis.

A central focus of research within the Branch continues to be the analysis of the synthesis, transport, and catabolism of the plasma lipoproteins in normal subjects and patients with dyslipoproteinemias.

ApoE has been extensively studied since it plays a pivotal role in lipoprotein metabolism. ApoE is a polymorphic protein, and there are three common isoproteins designated apoE<sub>2</sub>, apoE<sub>3</sub>, and apoE<sub>4</sub> in the population. ApoE<sub>3</sub> is the most common isoprotein, and is considered to be the normal isoprotein. We have previously established that apoE<sub>2</sub>, the major apoE isoprotein in patients with type III hyperlipoproteinemia, has delayed catabolism when compared to the catabolism of apoE<sub>3</sub>. Type III hyperlipoproteinemic patients also have reduced levels of LDL. In contrast, the apoE<sub>4</sub> isoprotein is more rapidly catabolized when compared to apoE<sub>3</sub>, and patients with apoE<sub>4</sub> have an elevated level of LDL. Based on LDL kinetics, the low level of LDL in type III patients, and the elevated levels of LDL in patients with the apoE<sub>4</sub> isoprotein are the results of increased and decreased rates of LDL catabolism, respectively. Based on these results we have proposed that the altered rates of catabolism of LDL in subjects with different apoE isoproteins is due to up and down regulation of the LDL receptor.

A new kindred with apoE<sub>1</sub> and type III hyperlipoproteinemia has been identified. Individual which are heterozygotes for the apoE<sub>1</sub> isoprotein have type III hyperlipoproteinemia or dysbetalipoproteinemia. Thus the presence of this unique apoE<sub>1</sub> variant designated apoE<sub>1</sub> Harrisburg (H), results in a dyslipoproteinemia with a dominant inheritance pattern. To elucidate the structural defect in apoE-1<sub>H</sub>, very low density lipoproteins (VLDL) were delipidated, and the apoE-1<sub>H</sub> was purified by heparin affinity chromatography. Peptides from apoE-1<sub>H</sub> were prepared by tryptic and cyanogen bromide digestion, and isolated by high pressure liquid and gel permeation chromatography, as well as fast peptide liquid chromatography. The isolated peptides were sequenced by automated Edman degradation, and the structural defect shown to be a glutamic acid for lysine substitution at residue 146. Kinetic analysis in normal volunteers revealed that apoE-1<sub>H</sub> has a significantly decreased fractional catabolic rate. These combined results have lead to the proposal that the mutant apoE-1 isoform not only is a nonfunctional apoE isoprotein, but also interferes with the physiological function of normal apoE<sub>3</sub> present on the lipoprotein particles. An understanding of the mechanism for this interesting dyslipoproteinemia will provide new insights into the physiological role of apoE in lipoprotein metabolism.

The role of apoA-I in high density lipoprotein (HDL) metabolism has been of major interest in the Branch. Three new apoA-I variants have been characterized. Subjects with apoA-I<sub>Milano</sub> (M) (residue 176 arg → cys) have low levels of apoA-I and HDL. ApoA-I<sub>M</sub> can form mono and





hetero-dimers due to the introduction of a cysteine in the amino acid sequence of apoA-I. Kinetic studies were performed to determine the metabolic reason for the low levels of plasma apoA-I and HDL in these patients. The catabolism of apoA-I<sub>M</sub> is complex with rapid catabolism of monomeric apoA-I<sub>M</sub> that also leads to rapid catabolism of normal apoA-I in heterozygotes since both apolipoproteins are on the same particle. In contrast the rate of catabolism of apoA-I<sub>M</sub> dimers is decreased. The levels of apoA-I<sub>M</sub> monomer and dimer, as well as normal apoA-I are due to the percentage of apoA-I<sub>M</sub> present in the plasma as monomer vs dimer. These studies have provided an explanation of the variable plasma levels of the apoA-I<sub>M</sub> isoproteins observed in these patients.

Probands with a second apoA-I variant, apoA-I<sub>Baltimore(B)</sub> have low levels of plasma apoA-I as well as HDL, and premature cardiovascular disease. ApoA-I<sub>B</sub> was purified from HDL from the proband, and sequence analysis revealed that the structural defect in apoA-I<sub>B</sub> is a leucine for an arginine substitution at residue 10. Kinetic analysis of apoA-I<sub>B</sub> in normal subjects and a proband with apoA-I<sub>B</sub> revealed that apoA-I<sub>B</sub> is catabolized at a normal rate in a normal subject while both apoA-I<sub>B</sub> and normal apoA-I are catabolized at an increased rate in apoA-I<sub>B</sub> subjects. Therefore, the low HDL levels in the apoA-I<sub>B</sub> subjects are due to either a generalized upregulation of the apoA-I catabolic pathway by apoA-I<sub>B</sub> or to apoB-I<sub>B</sub> being a marker for a closely related gene that upregulates the catabolism of HDL.

A new apoA-I variant, apoA-I<sub>Iowa(I)</sub>, has been shown to be a new cause of familial amyloidosis. ApoA-I<sub>I</sub> is the protein in tissue amyloid, and is the first recognized human apolipoprotein variant associated with amyloidosis. Sequence analysis of apoA-I<sub>Iowa</sub> revealed arginine for glycine substitution at position 26. The amino acid substitution in apoA-I<sub>I</sub> results in a 1+ charge shift in the isoelectrofocusing pattern of apoA-I and can now be used as a plasma test for diagnosis of asymptomatic individuals with familial amyloidosis. Current studies are underway to study the physical and metabolic properties of this unusual apoA-I.

During the last two years, studies have been conducted to develop the methodology for performing kinetic analysis of lipoprotein metabolism with a non-radioactive isotopic amino acid tracer. The non-radioactive isotopically labeled amino acids are infused as a bolus, and the labeled amino acid is incorporated into the individual plasma apolipoproteins. Plasma is obtained from the individual, the apolipoproteins isolated by high pressure liquid chromatography, the apolipoproteins hydrolyzed, and the infused labeled amino acids as well as the natural amino acids are quantitated by selective ion monitoring mass spectroscopy. Three normal subjects have been studied utilizing this techniques. The fractional catabolic rates of apoA-I, and apoE as well as VLDL, IDL, and LDL apoB were similar to data obtained from injected radiolabeled apoA-I, apoE, and apoB. These results provide data to indicate that this will be an extremely useful new technique for performing metabolic studies of apolipoproteins in humans.

One of the major challenges in the field of lipoproteins and atherosclerosis is the pathophysiological mechanisms involved in the



development of the atherosclerotic lesion. The study of cholesterol accumulation in the early atherosclerotic lesions has been of particular interest. Two cholesterol-rich lipid particles, one rich in unesterified cholesterol and the other rich in esterified cholesterol have been identified in the extracellular space of the human atherosclerotic lesions. The unesterified cholesterol (UC) rich lipid particle has a hydrated density of approximately 1.03 g/ml, a molar ratio of UC to phospholipid of 2.6:1, 76% of the cholesterol is UC, and sphingomyelin is the predominant phospholipid (PL). The cholesterol ester (CE) rich particle has a hydrated density of < 1.01 g/ml, 2.6:1 molar ratio of UC to PL, 72% of the cholesterol is CE, and the predominant phospholipid is sphingomyelin. Studies are underway to establish the origin of the particles, and the role that plasma lipoproteins play in the production and removal of these lipid complexes.

Of particular interest are studies undertaken to establish if HDL or isolated apoA-I are able to solubilize the UC in the lipoprotein particles. HDL but not apoA-I served as cholesterol acceptors effectively solubilizing cholesterol from the aortic particles. A separate series of studies are directed toward the elucidation of the role of UC/CE of LDL in the formation of the UC- rich lipid particles. Two model systems, type C Niemann-Pick disease cells and livers of NCTR-BALB/c mice which have defects in intracellular cholesterol processing and result in the accumulation of cellular UC are being used to study the formation and potential secretion of UC rich lipid particles. The role of activated platelets which induce cholesterol accumulation in cultured rat smooth muscle cells, and human fibroblasts is being investigated. The importance of platelet derived cholesterol in the atherosclerotic lesion has not been established, and may be of considerable importance to the underlying cause of the pathological process.

A major cell type in the development of atherosclerosis is the monocyte-macrophage. These cells play a central role in the development of the fatty streak, and the early vascular lesion in atherosclerosis. Cholesterol and triglyceride metabolism is of particular interest also in patients with Tangier disease, a disease characterized by an accumulation of CE in macrophage. The lipoproteins and apolipoproteins which modulate lipid accumulation of macrophages in normal cells and macrophage isolated from patients with dyslipoproteinemias are being actively studied. These combined studies will provide additional insights into the mechanisms involved in cholesterol accumulation in the atherosclerotic lesions, and may provide new approaches to the treatment of patients with premature cardiovascular disease.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02010-17 MDB

## PERIOD COVERED

October 1, 1987 through September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structure and Function of Plasma Lipoproteins and Apolipoproteins

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	H. Bryan Brewer, Jr., M.D.	Chief	MDB, NHLBI
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	A. Hospattankar, Ph.D.	Visiting Associate	MDB, NHLBI
	J. Hoeg, M.D.	Senior Investigator	MDB, NHLBI
	R. Ronan, B.A.	Chemist	MDB, NHLBI
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## COOPERATING UNITS (if any)

Dr. Dubo Bojanovski  
Zentrum Innere Medizin, Medizinische Hochschule Hannover  
Hannover, West Germany

## LAB/BRANCH

Molecular Disease Branch

## SECTION

Peptide Chemistry

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

5.9

## PROFESSIONAL:

3.9

## OTHER:

2.0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Human apoB isoforms have been analysed in human intestinal organ cultures in adults and children. The secreted apoB isoproteins were analysed by NaDodSO<sub>4</sub> gel electrophoresis and immunoblot analysis utilizing apoB monoclonal antibodies. The results of these studies established that the human intestine secretes both apoB-48 and apoB-100, with apoB-100 being the predominant apoB isoprotein secreted by the intestinal organ cultures in vitro. These results are of major importance since apoB-100 containing lipoproteins are known to be atherogenic.

Apolipoprotein variants have been isolated from patients with dyslipoproteinemias. ApoA-I<sup>Baltimore</sup> (arg -> leu) has been isolated from a kindred with familial hypoalphalipoproteinemia and premature cardiovascular disease. Purified apolipoprotein<sup>Baltimore</sup> has been utilized in kinetic studies which established that the amino acid substitution was not associated with any change in the metabolism of apoA-I. ApoE-1<sup>Harrisburg</sup> has been purified from a kindred with a dominant form of type III hyperlipidemia. The sequence of apoE<sub>1</sub><sup>Harrisburg</sup> was determined and shown to be a glutamic acid for lysine substitution at position 145. The metabolism of apoE-1<sup>Harrisburg</sup> was analyzed, and the catabolism of the apoE variant was markedly delay when injected into normal volunteers.

621



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02012-13 MDB

## PERIOD COVERED

October 1, 1987 through September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Zafarul H. Beg, Ph.D.	Research Chemist	MDB, NHLBI
J.A. Stonik	Chemist	MDB, NHLBI
H.B. Brewer, Jr., M.D.	Chief	MDB, NHLBI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Peptide Chemistry

## SECTION

Molecular Disease Branch

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2.0

## PROFESSIONAL:

1.0

## OTHER:

1.0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have previously established that rat and human hepatic HMG-CoA reductase activity is modulated in vitro and in vivo in a bicyclic cascade system involving reversible phosphorylation of both HMG-CoA reductase and reductase kinase. We have also demonstrated that enzymic activity of HMG-CoA reductase is also modulated in vitro by a protein kinase C-mediated phosphorylation. Recently we have demonstrated that HMG-CoA reductase activity is modulated by a third kinase system, a  $\text{Ca}^{2+}$  calmodulin dependent kinase, involving covalent phosphorylation. Recently in Hep G2 cells we have also investigated the short-term (reversible phosphorylation) and long-term (decreased protein synthesis) control of HMG-CoA reductase by utilizing ligands such as LDL and 25-hydroxycholesterol.

In order to understand the coordinate regulation of HMG-CoA reductase, cholesterol synthesis, and role of apolipoproteins such as apolipoprotein A-I (apoA-I) and apolipoprotein B (apoB) in the transport and regulation of cellular cholesterol, a systematic investigation of their role in plasma lipid and lipoprotein transport and metabolism has been undertaken. HDL have been proposed to transport excess cholesterol from peripheral cells back to the liver. Clinically, there is an inverse relationship between plasma apoA-I as well as HDL cholesterol levels and an increased risk of premature cardiovascular disease.

During the past year we have studied the post-translational modification of human plasma apoA-I involving reversible phosphorylation. In order to establish the physiological relevance of in vitro phosphorylation of human apoA-I, we have also demonstrated that secreted and intracellular apoA-I from Hep G2 cells were phosphorylated. The phosphorylation of apoA-I may play an important role in lipoprotein assembly, intracellular transport, as well as processing and lipoprotein secretion.

626





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02019-10 MDB

## PERIOD COVERED

October 1, 1987 through September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Metabolism of Lipoprotein and Apolipoproteins in Humans

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Richard E. Gregg, M.D.	Senior Investigator	MDB, NHLBI
Others: Paola Roma, Ph.D.	Visiting Fellow	MDB, NHLBI
Robert Ross, M.D.	Medical Staff Fellow	MDB, NHLBI
Juergen Schaefer, M.D.	Visiting Fellow	MDB, NHLBI
Alexander Mann, M.D.	Visiting Fellow	MDB, NHLBI
Diana Hernandez	Chemist	MDB, NHLBI
Marie Kindt	Chemist	MDB, NHLBI
Robert Herzog	Biological Aid	MDB, NHLBI

## COOPERATING UNITS (if any)

Loren A. Zech, M.D.	Senior Investigator	Office of Dir., NHLBI
Dubo Bojanovski, M.D., Ph.D.	Assistant Professor	Univ. Hannover, FRG
Merril Benson, M.D.	Professor	Indiana Univ.

## LAB/BRANCH

Molecular Disease Branch

## SECTION

Peptide Chemistry

## INSTITUTE AND LOCATION

NIH, NHLBI, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

6.5

## PROFESSIONAL:

4

## OTHER:

2.5

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Apolipoprotein (apo) E is a genetically polymorphic protein with three common isoforms in humans. We have identified a kindred with a mutant apoE, apoE-I<sup>Harrisburg</sup>, in which individuals heterozygous for this mutant allele have type III HLP. The mutation is a lysine to glutamic acid substitution at A.A. 146. This mutation results in a decreased fractional catabolic rate for apoE and presumably for apoB containing lipoprotein remnants resulting in type III HLP.

The kinetics of metabolism of mutant forms of apoA-I have been investigated. Individuals with apoA-I<sup>Milano (M)</sup> have low levels of apoA-I and HDL. ApoA-I<sup>M</sup>, which can form disulfide linked dimers, has a very complex metabolic pathway with rapid catabolism of monomeric apoA-I<sup>M</sup> that also results in rapid catabolism of normal apoA-I, and slow catabolism of apoA-I<sup>M</sup> dimers. This results in low plasma apoA-I and HDL levels with approximately equal amounts of apoA-I<sup>M</sup> and normal apoA-I in these heterozygotes. ApoA-I<sup>Baltimore (B)</sup> subjects have low HDL levels with premature atherosclerosis. ApoA-I<sup>B</sup> is catabolized at a normal rate in a normal subject while both apoA-I<sup>B</sup> and normal apoA-I are catabolized at an increased rate in apoA-I<sup>B</sup> subjects. Therefore, the low HDL levels in the apoA-I<sup>B</sup> subjects are due either to a generalized upregulation of the apoA-I catabolic pathway by apoA-I<sup>B</sup> or to apoA-I<sup>B</sup> being a marker for a closely linked gene that upregulates the catabolism of apoA-I and HDL.

Kinetic studies of apolipoprotein metabolism have been performed in three normal subjects utilizing nonradioactive heavy isotope labeled amino acids administered by bolus injection. There were no alterations in the metabolism of simultaneously administered radiolabeled apoA-I and apoB, and the catabolic rates of nonradioactive heavy isotope labeled apoA-I, apoE, as well as VLDL, IDL, and LDL apoB were similar to results for radiolabeled studies of these apolipoproteins. This will be a useful new method to perform metabolic studies of apolipoproteins in humans.

632



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02022-08 MDB

## PERIOD COVERED

October 1, 1987 through September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cellular Lipid and Lipoprotein Biochemistry

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Jeffrey M. Hoeg, M.D.	Senior Investigator	MDB, NHLBI
Thomas Eggerman, MD, Ph.D.	Medical Staff Fellow	MDB, NHLBI
Hans-Joerg Kraft, Ph.D.	Visiting Scientist	MDB, NHLBI
Christopher JN Rall,	HH Medical Student	MDB, NHLBI
Stephen J. Demosky, Jr.	Chemist	MDB, NHLBI
Barbara Winterrowd	Medical Technician	MDB, NHLBI
Tracy Wahl	Medical Technician	MDB, NHLBI
Uwe K. Schumacher	Biologist	MDB, NHLBI

## COOPERATING UNITS (if any)

Drs. Louisa Laue, William Gahl, and George Chrousos, NICHD  
Drs. V Repin, D Sviridov, V Kosykh, V Smirnov, Cardiocenter, USSR

## LAB/BRANCH

Molecular Disease Branch

## SECTION

Peptide Chemistry

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

## PROFESSIONAL:

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☒ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The principal interest of this laboratory is to evaluate cellular lipoprotein and apolipoprotein metabolism. Several different human cells lines are maintained in tissue culture and the ability of cells, lipoproteins, and apolipoproteins from normolipidemic and dyslipidemic patients are studied. Our previous investigations on receptors for low density lipoproteins and high density lipoproteins and the intracellular enzymes relevant to cellular cholesterol homeostasis (acid and neutral cholesteryl ester hydrolase, Acyl: cholesterol acyltransferase, and HMG-CoA reductase) have been extended to evaluate the coordinate regulation of these proteins in human hepatocytes and in the human hepatoma cell line Hep G2. Since the liver is a primary source of apolipoprotein synthesis as well as lipoprotein catabolism, these studies are now focused upon how these different intracellular pathways are related to nascent apolipoprotein biogenesis. Apolipoprotein synthesis is regulated at the levels of transcription and translation. In addition, several of the apolipoproteins are modified post-translationally by glycosylation, fatty acid acylation, co- and post-translational proteolytic cleavage, and by phosphorylation. Insights gained into apolipoprotein synthesis and structure-function relationships are complemented by outpatient clinical trials designed to modify apolipoprotein synthesis and secretion in hyperlipidemic patients.

639



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02028-04 MDB

PERIOD COVERED October 1, 1987 through September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
Molecular Biology of the ApoC-II Gene

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Silvia S. Fojo, Ph.D., M.D. Senior Staff Fellow MDB, NHLBI  
Others: H. Bryan Brewer, Jr., M.D. Chief MDB, NHLBI

## COOPERATING UNITS (if any)

Carlo Gabelli, M.D., and Giovannella Baggio, M.D. - University of Padova,  
Padova, Italy, Ulrike Beisiegel- University of Hamburg, Hamburg, West  
Germany

## LAB/BRANCH

Molecular Disease Branch

## SECTION

Peptide Chemistry

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

2.0

## PROFESSIONAL:

2.0

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have studied the genetic defect that leads to deficiency of apoC-II in e patients with apoC-II deficiency from independent kindreds

The first patient from Hamburg, West Germany, was found to have a single base substitution (guanosine for a cytosine,) at the first base of the donor splice site of intron II of the apoC-II gene by sequence analysis. This mutation should abolish normal splicing at this site and ultimately lead to the deficiency of plasma apoC-II observed in this kindred. Analysis of total RNA isolated from the patients liver by Northern, slot blot, and in situ RNA hybridization, as well as evaluation of the intrahepatic apoC-II content by immunohistochemistry confirmed the results of our sequence analysis.

The second patient from Nijmegen, the Netherlands was found to have a deletion of a cytosine at position # 2943 of exon 3 of the apoC-II gene. This mutation results in the formation of a premature termination codon at a position corresponding to amino acid #18 of normal apoC-II. As a result, this mutation leads to the formation of a truncated apoC-II that is unlikely to activate lipoprotein lipase and thus, leads to apoC-II deficiency in this kindred.

Total RNA isolated from the liver of a third patient with apoC-II deficiency was analyzed by Northern, slot blot and in situ RNA hybridization which revealed approximately normal levels of a normal sized apoC-II message. Immunohistochemical analysis showed normal to increased amounts of intrahepatic C-II apolipoprotein. We postulate that the defect in this patient from Padova with normal intrahepatic apoC-II mRNA and protein, but deviciency of apoC-II in plasma is a mutation in the coding portion of the apoC-II gene that results in abnormal secretion or enhanced catabolism of apoC-II.

644



## NOTICE OF INTRAMURAL RESEARCH PROJECT

201 HL 02029-01 MDB

## PERIOD COVERED

October 1, 1987 through September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular biology of plasma apolipoproteins and lipoproteins

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Keiichi Higuchi, Ph.D.	Visiting Fellow	MDB, NHLBI
Ashok Hospattankar, Ph.D.	Visiting Associate	MDB, NHLBI
Gregory Tennyson, M.D.	Medical Staff Fellow	MDB, NHLBI
Nancy Meglin	Chemist	MDB, NHLBI
Charles Sabatos	Chemist	MDB, NHLBI
Thomas Eggerman, M.D.	Medical Staff Fellow	MDB, NHLBI
H. Bryan Brewer, M.D.	Chief	MDB, NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Molecular Disease Branch

## SECTION

Peptide Chemistry

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

6.3

## PROFESSIONAL:

4.3

## OTHER:

2.0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The structural relationship of human apoB-100 and apoB-48 have been elucidated by sequence analysis of cDNA clones of apoB-100 mRNA. Clones were obtained which contained a C -> T substitution at nucleotide 6666 in the apoB-100 cDNA. The C -> T substitution converted the 'CAA' codon for glutamine in apoB-100 into a 'TAA' premature stop codon. The change in the 'CAA' codon for glutamine for the 'TAA' stop codon resulted in the termination of translation at amino acid 2152. Therefore apoB-100 contained 4536 amino acids, and apoB-48 contained 2152 amino acids. The C -> T change was not present at the DNA level, and the mechanism for the production of apoB-48 is a unique RNA editing mechanism which has not been previously described.

The RNA editing mechanism for apoB-48 biosynthesis was shown to be present in both the liver and intestine in the human as well as the rat.

A detailed analysis of the 5' regulatory region of the apoA-I gene has been performed. TATA box, CAT box like, and two GC sequences were identified. Both promoters as well as tissue specific repressors of apoA-I expression in non-apoA-I producing cells were present in the 5' flanking region of the apoA-I gene.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02030-01 MDB

## PERIOD COVERED

October 1, 1987 through September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Interaction of HDL with vascular-derived cholesterol-rich lipid particles

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Ya-Jun Chen Guest Worker MDB, NHLBI

Others: Fei-Fei Chao Visiting Associate MDB, NHLBI  
Howard S. Kruth Chief, Sect. of Exp. Athero. MDB, NHLBI

## COOPERATING UNITS (if any)

Laboratory of Cellular and Developmental Biology, NIDDK (E.J. Blanchette-Mackie and N. Dwyer); Department of Pathology, University of Maryland, School of Medicine (J. Resau and W.T. Mergner)

## LAB/BRANCH

Molecular Disease Branch

## SECTION

Section of Experimental Atherosclerosis

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

## PROFESSIONAL:

## OTHER:

0.5

0.5

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have previously identified and isolated unesterified cholesterol-rich particles that accumulate in the extracellular space of human atherosclerotic lesions. HDL is the major lipoprotein class thought to be involved in transporting cholesterol from peripheral tissues to the liver for reutilization or excretion. The purpose of this project is to determine whether and by what mechanism human high density lipoprotein (HDL) can solubilize these unesterified cholesterol-rich lipid particles.

In order to study the interaction between aortic unesterified cholesterol-rich particles (diameter = 1000 Å) and HDL (diameter = 100 Å), we incubated the aortic particles with HDL and studied the morphological changes in these particles. As a result of incubation, a new intermediate sized particle appeared with a modal diameter of 180-200 Å (size range between 150-250 Å). These new lipid particles surrounded the larger aortic particles or occupied the entire domain of the aortic particles. This suggested that HDLs transformed the large aortic particles into smaller intermediate sized particles or that HDLs served as cholesterol acceptors effectively solubilizing cholesterol from the aortic particles. With solubilization of cholesterol, HDL transformed into an enlarged cholesterol-rich HDL.

Unesterified cholesterol-rich lipid particles have been shown to be an important early event in the development of atherosclerotic lesions. Removing these particles from the atherosclerotic lesion, therefore, could be a way to reduce the atherosclerosis process. Our preliminary findings suggest that plasma HDL may contribute to the removal of these atherosclerotic plaque lipid particles. Further investigation concerning mechanisms of this reaction may help elucidate ways to enhance lipid removal from atherosclerotic lesions.

654



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02031-01 MDB

## PERIOD COVERED

October 1, 1987 through September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cholesterol metabolism in human monocyte-derived macrophages

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Sonia I. Skarlatos Staff Fellow MDB, NHLBI

Others: Rani Rao Chemist MDB, NHLBI  
George Johnson Biol. Lab. Tech. MDB, NHLBI  
Robert Herzog Biological Aid MDB, NHLBI  
Frances Carter Biol. Lab. Tech. MDB, NHLBI  
H. Bryan Brewer Chief MDB, NHLBI  
Howard S. Kruth Chief, Sect. of Exp. Athero. MDB, NHLBI

## COOPERATING UNITS (if any)

Department of Transfusion Medicine, CC

## LAB/BRANCH

Molecular Disease Branch

## SECTION

Section of Experimental Atherosclerosis

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1

## PROFESSIONAL:

0.5

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The purpose of this project is to study cholesterol metabolism in human monocyte-derived macrophages, a major cell that accumulates cholesterol in atherosclerotic lesions. Also, because cholesterol accumulates predominantly in macrophages in genetically determined cholesterol storage diseases, we plan to use cultured human monocyte-derived macrophages from patients with these diseases to investigate possible cellular abnormalities in lipoprotein and cholesterol processing in these diseases.

When normal monocyte-derived macrophages were incubated with low density lipoprotein, macrophages accumulated unesterified cholesterol (15 nM/mg protein) but did not accumulate triglycerides and usually no cholesteryl ester. Addition of albumin(0.35%) potentiated the accumulation of unesterified cholesterol by macrophages. When normal monocyte-derived macrophages were incubated with very low density lipoprotein (VLDL), macrophages accumulated triglycerides but did not accumulate cholesteryl ester. Triglyceride accumulation in normal monocyte-derived macrophages increased proportionately with increasing concentrations of VLDL. Accumulation of triglycerides by macrophages incubated with VLDL did not depend on the presence of serum. When macrophages were incubated with VLDL at total cholesterol concentrations below and above 150 nM/ml, cellular unesterified cholesterol content decreased. VLDL added at a concentration of 150 nM/ml stimulated cholesterol accumulation of unesterified cholesterol. When macrophages were incubated with 50% or 100% human serum total cellular cholesterol content decreased 28% and 45%, respectively.

Because macrophages are one of the major types of cells which accumulate cholesterol in atherosclerotic lesions, it is of great significance to develop an in vitro model to study their cholesterol metabolism. By incubating these cells with lipoprotein and non-lipoprotein cholesterol forms, we will be able to examine pathways of cholesterol metabolism in macrophages from normal individuals and patients with cholesterol storage diseases.

657



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02032-01 MDB

## PERIOD COVERED

October 1, 1987 through September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Development of unesterified cholesterol-rich lipid particles in cultured cells

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Fei-Fei Chao Visiting Associate MDB, NHLBI

Others: Ya-Jun Chen Guest Worker MDB, NHLBI

George Johnson Biol. Lab. Tech. MDB, NHLBI

Howard S. Kruth Chief, Sect. of Exp. Athero. MDB, NHLBI

## COOPERATING UNITS (if any)

Laboratory of Cellular and Developmental Biology, NIDDK (E.J. Blanchette-Mackie);  
Developmental and Metabolic Neurology Branch, NINCDS (P.G. Pentchev and M. Comly)

## LAB/BRANCH

Molecular Disease Branch

## SECTION

Section of Experimental Atherosclerosis

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

## PROFESSIONAL:

0,5

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☒ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The purpose of this project is to explore the possibility that low density lipoprotein (LDL), once taken up by cells, can be degraded and transformed into unesterified cholesterol-rich lipid particles similar to those that we have previously identified and isolated from atherosclerotic lesions (see report Z01 HL 02826-07 EA). Two model systems, cultured fibroblasts from patients with type C Niemann-Pick disease (NPC) and livers of NCTR-BALB/c mice, were chosen for initial studies of cellular production and accumulation of unesterified cholesterol-rich lipid particles. Both models are autosomal-recessive lipid storage disorders with defects in intracellular cholesterol processing resulting in accumulation of unesterified cholesterol rather than esterified cholesterol, the usual storage form of excess cellular cholesterol.

Unesterified cholesterol-containing inclusions accumulated within cultured NPC fibroblasts during incubation with LDL. These inclusions appeared to be multilamellar liposomes with a hydrated density between 1.03 g/ml and 1.06 g/ml. The liposomes contained most of their cholesterol in an unesterified form (>90%) with a 1.5:1 molar ratio of unesterified cholesterol to phospholipid. NPC fibroblasts and normal fibroblasts incubated with lipoprotein-deficient serum plus LDL did not accumulate similar liposomes. This indicates that these unesterified cholesterol-containing liposomes were specifically derived from degradation of LDL and abnormal processing of cholesterol released from degraded LDL. Unesterified cholesterol-containing vesicles accumulated in livers of NCTR-BALB/c mice. These vesicles appeared to be uni- and multilamellar structures with a hydrated density between 1.04-1.07 g/ml. Most of their cholesterol was also in an unesterified form (>90%) with a 2.5:1 molar ratio of unesterified cholesterol to phospholipid.

Studies carried out with both models should be helpful in elucidating the mechanism of formation of the unesterified cholesterol-rich lipid particles that accumulate within atherosclerotic lesions.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02826-07 MDB

## PERIOD COVERED

October 1, 1987 through September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Isolation and characterization of lipid-rich particles in atherosclerotic lesions

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Fei-Fei Chao	Visiting Associate	MDB, NHLBI
Others:	Ya-Jun Chen	Guest Worker	MDB, NHLBI
	Sonia I. Skarlatos	Staff Fellow	MDB, NHLBI
	Howard S. Kruth	Chief, Sect. of Exp. Athero.	MDB, NHLBI

## COOPERATING UNITS (if any)

Lab. of Cell. & Devel. Biology, NIDDK (E.J. Blanchette-Mackie and N. Dwyer); Dept. of Physiology, George Washington University (B.F. Dickens); Dept. of Nutrition, USDA (E. Berlin); Dept. Path., Univ. of Md., Sch. of Med. (J. Resau & W.T. Mergner)

## LAB/BRANCH

Molecular Disease Branch

## SECTION

Section of Experimental Atherosclerosis

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2

## PROFESSIONAL:

1

## OTHER:

1

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have identified two cholesterol-rich lipid particles, one rich in unesterified cholesterol and the other rich in esterified cholesterol in the extracellular space of human atherosclerotic lesions. The purpose of this project is to study the chemical and structural properties of these two cholesterol-rich particles and to determine their roles in vascular lipid deposition.

The unesterified cholesterol-rich lipid particles have a hydrated density of 1.02-1.06 g/ml (peak at 1.036 g/ml). They have a high molar ratio of unesterified cholesterol to phospholipid (2.6:1) and 76% of particle cholesterol is unesterified. The esterified cholesterol-rich particles have a hydrated density of less than 1.01 g/ml. These particles also have a 2.6:1 molar ratio of unesterified cholesterol to phospholipid, but 72% of particle cholesterol is esterified. The phospholipid classes and cholesteryl ester fatty acid composition of the two lipid particles are similar. The predominant phospholipids are sphingomyelin(55%) and phosphatidylcholine(27%). Both particles contain substantial amounts of lysophosphatidylcholine(7-9%). Phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine are present in trace amounts. The major fatty acids in lipid particle cholesteryl esters are palmitate(20%), oleate(30%), and linoleate(30%). Both particles have diameters between 70 and 300 nm. The unesterified cholesterol-rich particles are spherical and vesicular and show uni- and multilamellar structures. The esterified cholesterol-rich particles are very small lipid droplets often demonstrating pitting (possible sites of lipolysis) at their surface.

The two cholesterol-rich particles we have isolated appear to represent an early pathologic form of cholesterol that accumulates in the vessel wall during atherogenesis. Studies of the origin and fate of these particles will contribute to a better understanding of the atherosclerotic disease process.

664





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02828-03 MDB

## PERIOD COVERED

October 1, 1987 through September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Platelet-mediated cellular cholesterol accumulation

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Sonia I. Skarlatos Staff Fellow MDB, NHLBI

Others: George Johnson Biol. Lab. Tech. MDB, NHLBI  
Frances Carter Biol. Lab. Tech. MDB, NHLBI  
Rani Rao Chemist MDB, NHLBI  
Howard S. Kruth Chief, Sect. of Exp. Athero. MDB, NHLBI

## COOPERATING UNITS (if any)

Department of Transfusion Medicine, CC  
Section of Laboratory Animal Medicine and Surgery, NHLBI

## LAB/BRANCH

Molecular Disease Branch

## SECTION

Section of Experimental Atherosclerosis

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1

## PROFESSIONAL:

0.5

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

From our previous studies we determined that activated rat platelets induce cholesterol accumulation in cultured rat smooth muscle cells and human fibroblasts, whereas, activated human platelets do not. We would like to establish the mechanism of platelet-mediated cellular cholesterol accumulation and the basis for the differential response with human and rat platelets. In this study we have examined changes in platelet phospholipids induced by activation to determine if this could be a factor in platelet-mediated cholesterol accumulation.

We have observed that activation of rat platelets results in significant phospholipid hydrolysis (80% of initial values). This results in an increased cholesterol to phospholipid molar ratio and could potentially promote redistribution of cholesterol from the platelets to other cells or blood lipoproteins. The change in phospholipid content observed in activated rat platelets may represent a more generalized mechanism to promote cholesterol removal from dying cells. When rat platelets were activated they released significant lactate dehydrogenase suggesting that the platelets were dying. By contrast, when human platelets were activated they showed no significant change in phospholipid content or C/P ratio nor did they release nearly as much lactate dehydrogenase as rat platelets. This suggests that the human platelets remained viable after activation.

Further studies of platelet-mediated cellular cholesterol accumulation should help elucidate the mechanism(s) of cholesterol removal from cells that die in atherosclerotic lesions and thrombi associated with atherosclerotic lesions. The cholesterol burden from dying cells may contribute significantly to the overall accumulation of cholesterol within atherosclerotic lesions.

667



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02829-02 MDB

## PERIOD COVERED

October 1, 1987 through September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structural changes of human LDL accompanying hydrolysis of cholesteryl ester

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Fei-Fei Chao Visiting Associate MDB, NHLBI

Others: Ya-Jun Chen Guest Worker MDB, NHLBI  
Howard S. Kruth Chief, Sect. of Exp. Athero. MDB, NHLBI

## COOPERATING UNITS (if any)

Laboratory of Cellular and Developmental Biology, NIDDK (E.J. Blanchette-Mackie and N. Dwyer)

## LAB/BRANCH

Molecular Disease Branch

## SECTION

Section of Experimental Atherosclerosis

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2

## PROFESSIONAL:

1

## OTHER:

1

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The objective of this project is to determine whether the major cholesteryl ester-rich lipid particle in plasma, low density lipoprotein (LDL), can transform by treatment with cholesterol esterase into a larger unesterified cholesterol-rich particle similar to those we have isolated from atherosclerotic lesions.

When LDL was incubated with cholesterol esterase, the LDL cholesteryl ester was completely hydrolyzed during 4 hrs of incubation. Interestingly, only when LDL was treated with trypsin prior to incubation with cholesterol esterase was LDL cholesteryl ester hydrolyzed. When LDL was incubated in a weak buffer in which pH gradually changed from 7.2 to 6.0 during the incubation, the LDL cholesteryl ester was initially hydrolyzed from 75% to 12% (molar % of total cholesterol). However, upon prolonged incubation, a reverse reaction occurred in which re-esterification of cholesterol slowly proceeded. After 24 hrs of CEase incubation, the degraded LDL contained 55% of its cholesterol as ester. The addition of human serum albumin prevented cholesterol re-esterification indicating that removal of free fatty acids from hydrolyzed LDL attenuated the cholesterol re-esterification process probably due to lack of substrate.

The structure of the LDL particle changed following hydrolysis of LDL cholesteryl ester. Small projections extended from the surface of LDL during early cholesteryl ester hydrolysis. These projections transformed into films of various sizes and shapes, some still associated with the native LDL. With complete hydrolysis, flattened vesicles of irregular shape and aggregates of flat or curled films were present. In LDL incubated with the weak buffer the cholesterol re-esterification resulted in disruption of the films and the formation of lipid droplet-like spherical particles. Our results demonstrate that LDL transforms into a larger vesicular structure during hydrolysis of its cholesteryl ester core. This newly-formed larger lipid particle may become trapped in the vessel wall and lead to vascular cholesterol accumulation. 670



Annual Report of the  
Laboratory of Molecular Hematology  
National Heart, Lung, and Blood Institute  
October 1, 1987 to September 30, 1988

The Laboratory of Molecular Hematology (LMH) is composed of three sections: the Section on Molecular Genetics is primarily involved in developing the basic knowledge and technology for carrying out gene therapy for human genetic diseases; the Section on Molecular Cloning is primarily concerned with understanding the nature of transcriptional control elements; and the Section on RNA and Protein Biosynthesis is primarily concerned with understanding the mechanism and regulation of eukaryotic gene expression at both the transcriptional and translational levels.

SECTIONS ON MOLECULAR GENETICS AND MOLECULAR CLONING

The diseases chosen as the initial candidates for human gene therapy are adenosine deaminase (ADA) deficiency, a cause of severe combined immunodeficiency (SCID), and advanced cancer. Retroviral techniques and recombinant DNA technology have been used to construct retroviral vectors containing the human ADA gene as well as a selectable gene, NeoR (the latter codes for a phosphotransferase enzyme that confers resistance to the drug G418, a neomycin analogue that can kill mammalian cells). A highly efficient procedure for transferring functional genes into mammalian tissue culture cells in vitro and into bone marrow cells of mice in vivo has been developed over past years using these retroviral vectors as a delivery system.

Previously we demonstrated that when murine hematopoietic progenitor cells are infected in vitro with a vector carrying the NeoR gene and then reinjected into a lethally irradiated recipient mouse, 85-90% of the stem cells (CFU-S) can be shown to carry an intact copy of the NeoR gene. The majority of these cells can be shown (by analyzing spleen foci in the CFU-S assay) to produce the NeoR phosphotransferase (NPT). Using the knowledge gained from the murine system, an autologous bone marrow transplantation (BMT)/gene transfer protocol was developed for nonhuman primates. These results have now been extended to several additional cell types: tumor infiltrating lymphocytes (TIL), endothelial cells, hepatocytes, and fibroblasts.

During the past year, these two Sections have achieved the following results:

(1) Human tumor infiltrating lymphocytes (TIL) have been successfully infected with the N2 vector containing the Neo<sup>R</sup> gene. A clinical protocol has been developed with Dr. Steven A. Rosenberg, Chief, Surgery Branch, NCI, and Dr. R. Michael Blaese, Chief, Cellular Immunology Section, Metabolism Branch, NCI, which is designed to provide information on the trafficking of TIL during adoptive immunotherapy of patients with advanced cancer. The protocol has been approved by the NHLBI and NCI IRBs and by the NIH Institutional Biosafety Committee. If finally approved by the NIH Recombinant DNA Advisory Committee (RAC), it would be the first use of gene transfer technology in human patients.

(2) The technology has been developed for inserting genes into hepatocytes, growing the gene-engineered cells on three-dimensional collagen-coated pads, and



inserting the cell-containing pads into animals either into subcutaneous or intraperitoneal sites. Hepatocytes can be recovered from the pads after two weeks in vivo that still express the inserted gene.

(3) Vascular endothelial cells have successfully been used for the insertion of genes which produce secretable proteins. The gene-engineered endothelial cells have been grown on vascular grafts and shown to continue to secrete the gene product in vitro. In vivo studies are being initiated.

#### SECTION ON RNA AND PROTEIN BIOSYNTHESIS

To understand the regulation of expression of genes transcribed by RNA polymerase II, promoter elements of the Adenovirus 2 major late transcription unit and the eukaryotic translation factor eIF-2 are being used to characterize and purify individual factors required for correct initiation and regulation of transcription.

The mechanisms by which adenoviruses and influenza viruses take over the translational machinery of the infected cell, and the defense mechanisms used by cells to prevent viral takeover are being studied.

The role of eIF-2B during protein synthesis initiation in normal and viral infected cells is being studied using antibodies directly against eIF-2B and factors whose activities it modulates.

During the past year this Section has achieved the following results:

(1) The promoter region of the eIF-2 $\alpha$  gene has been sequenced and analyzed. This gene is characterized by an unusual 4 kb long intron within the 5'-UTR. The promoter region of this housekeeping gene contains neither a TATA box nor a CAAT box. By S1 nuclease and primer extension analysis, 10 to 12 transcription start sites are identified within a 43 bp region.

(2) The eIF-2 $\alpha$  promoter region has been examined by in vivo and in vitro footprinting, as well as consensus binding site analysis. Although several previously characterized binding sites such as those for AP-1 and Sp1 are identified, 6 new binding sequences are found. Their effects on transcriptional activity of the eIF-2 $\alpha$  gene are under investigation.

(3) Several new promoter elements for the Adenovirus 2 major late promoter have been identified by DNA-affinity techniques and functional transcription assays. One (DTF) binds to adownstream promoter sequence extending from +146 to +165 (relative to the cap site at +1). A second binds just downstream of the TATAA factor binding site. In addition, several specific topoisomerase I binding sites map within the Ad2 MLP.

(4) By UV cross-linking, the DPS binding factor is identified as a new 40kDa transcription factor. In vivo functional analysis of DPS mutations introduced into the intact Ad2 genome is currently being analyzed.

(5) The mechanisms by which influenza virus prevents shutoff of protein synthesis by the eIF-2 $\alpha$  specific protein kinase activated during viral infection was studied. Influenza virus was found to encode a gene product which directly blocks kinase autophosphorylation and kinase activity. Suppression of kinase





activity occurs within 2 hours post-influenza infection and requires viral gene expression. In vitro mixing experiments also show that the influenza viral inhibition can act in trans to block kinase activity.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 HL 02213-11 MH
PERIOD COVERED October 1, 1987 through September 30, 1988		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) RNA Polymerase II Transcription Factors		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI: B. Safer, Medical Officer, LMH, NHLBI Others: R. Cohen, Staff Fellow, LMH, NHLBI W.F. Anderson, Chief, LMH, NHLBI T. Silverman, Staff Fellow, LMH, NHLBI W. Jacob, Staff Fellow, LMH, NHLBI J.A. Thompson, Expert, LMH, NHLBI S. Garfinkel, Biol. Lab. Tech., LMH, NHLBI T. Boal, Biol. Lab. Tech., LMH, NHLBI L. Yang, Biologist, LMH, NHLBI		
COOPERATING UNITS (if any) Michael Katze, U of Washington Medical School, Seattle, WA; Tom Shenk, Princeton University, Princeton, NJ; Rosemary Jagus, University of Pittsburgh, Pittsburgh, PA; John Hershey, University of California, Davis, CA.		
LAB/BRANCH Laboratory of Molecular Hematology		
SECTION Section on RNA and Protein Biosynthesis		
INSTITUTE AND LOCATION NHLBI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 6.3	PROFESSIONAL: 4.2	OTHER: 2.1
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Regulation of gene expression occurs at the level of <u>transcription</u>, <u>process-</u>  <u>ing</u>, <u>transport</u>, and <u>mRNA translation</u>. The primary goal of this section is to in-          vestigate the transcriptional and translational control mechanisms responsible for          regulated gene expression.</p> <p>To identify components required for transcription of genes by <u>RNA polymerase</u>  <u>II</u>, transcriptionally active nuclear extracts were fractionated using FPLC and DNA          affinity chromatography. Sequence-specific DNA binding factors required for tran-          scription of the adenovirus 2 major late promoter and the eIF-2<math>\alpha</math> promoter were          identified by DNase I footprinting, mobility shift assays, and their function(s)          studied using a novel assay system based on binding to synthetic oligonucleotides          of their cognate promoter sequences. In addition, a UV protein-DNA crosslinking          procedure was developed which is able to directly identify sequence specific DNA-          binding factors at very early stages of their purification. Using these methods,          trans acting factors binding to new Ad2 MLP promoter elements in addition to those          recognizing the TATAA and upstream promoter sequence (UPS) have been identified.          Characterization of the eIF-2<math>\alpha</math> promoter also identified new cis-acting promoter          elements.</p> <p>During infection by adenoviruses and influenza viruses, activation of host          cell ds-RNA dependent eIF-2<math>\alpha</math> kinase is prevented by VA1 RNA and an unknown, but          functionally similar, influenza gene product. The mechanisms by which certain cell          lines escape viral takeover of their translational factors which participate in          virus-host interactions, the genes for subunits of eIF-2 and eIF-2B are being          identified and sequenced. In addition, binding of VA1 RNA to interferon-induced          P68 eIF-2<math>\alpha</math> kinase has been shown to inhibit activation by double-stranded RNA.</p>		



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02216-09 MH

## PERIOD COVERED

October 1, 1987 through September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Correction of Genetic Defects by Gene Transfer

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: W. French Anderson, Chief, LMH, NHLBI (VA=Visiting Assoc.; MSF=Med. Staff Fellow)

## Others:

M. Eglitis, Guest Worker, LMH, NHLBI	S. Sturm, VA, LMH, NHLBI	D. Muenchau, Med. Tech
J. McLachlin, VA, LMH, NHLBI	J. Zwiebel, MSF, LMH, NHLBI	LMH, NHLBI
S. Bernstein, Biol., LMH, NHLBI	K. Karson, MSF, LMH, NHLBI	N. Nguyen, Med. Tech.,
R. Moen, Guest Worker, LMH, NHLBI	R. Wieder, MSF, LMH, NHLBI	LMH, NHLBI
S. Freeman, Guest Worker, LMH, NHLBI	J. Selegue, Biol., LMH, NHLBI	M. Daucher, Biol., LMH
K. Cornetta, NRSA Fellow, LMH, NHLBI	R. Morgan, MSF, LMH, NHLBI	NHLBI

## COOPERATING UNITS (if any)

A. Nienhuis, CHB, NHLBI; M. Blaese, MET, NCI; S. Rosenberg, SB, NCI; E. Zanjani, VA Hospital, Minn., MN; M. Harrison, U of CA Medical School, San Francisco, CA.

G. Springett, Howard Hughes Stud., LMH, NHI

## LAB/BRANCH

Laboratory of Molecular Hematology

## SECTION

Section on Molecular Genetics

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

12.8

## PROFESSIONAL:

8.2

## OTHER:

4.6

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A highly efficient procedure for transferring functional genes into mammalian cells has been developed using retroviral vectors as a delivery system. Retroviral vectors containing the human gene for the enzyme adenosine deaminase (ADA) as well as the NeoR gene have been made. Using the knowledge obtained from murine systems a non-human primate autologous bone marrow transplantation/gene transfer protocol has been developed. Low levels of both the human ADA gene and the NeoR gene have been expressed in the peripheral blood cells of several monkeys. In addition, these vectors have been used to introduce exogenous genes into human tumor infiltrating lymphocytes (TIL). These studies are preliminary to attempting human gene therapy in patients suffering from ADA severe combined immunodeficiency disease and advanced cancer.

6 p/



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02217-03 MH

## PERIOD COVERED

October 1, 1987 through September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Gene Expression Utilizing Nucleic Acid Manipulations

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J.A. Thompson, Guest Worker, LMH, NHLBI

Others: J. DiPietro, Biologist, LMH, NHLBI

K. Anderson, Guest Worker, LMH, NHLBI

W.F. Anderson, Chief, LMH, NHLBI

## COOPERATING UNITS (if any)

L. Reid, Albert Einstein College of Medicine, Bronx, NY; T. Maciag, American Red Cross, Rockville, MD.

## LAB/BRANCH

Laboratory of Molecular Hematology

## SECTION

Section on Molecular Cloning

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2.1

## PROFESSIONAL:

1.1

## OTHER:

1.0

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Rat hepatocytes have been used to develop the technology for carrying out gene transfer into cells of solid organs. The NeoR gene has been inserted into hepatocytes which are grown on a three-dimensional collagen-coated pad. The pad has been implanted into subcutaneous and intraperitoneal sites in rats. Two weeks later the pads were removed and shown to still contain hepatocytes expressing the inserted gene.

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Annual Report of the Pathology Branch  
Division of Intramural Research  
National Heart, Lung, and Blood Institute  
October 1, 1987, to September 30, 1987

As in the past years, studies focused on morphologic aspects of coronary, myocardial, and congenital heart diseases.

CORONARY ARTERY DISEASE

During recent years, several investigators examined at necropsy the degrees of cross-sectional area (CSA) luminal narrowing in the major epicardial coronary arteries in fatal coronary arterial disease. Recently, we switched from determining degrees of luminal narrowing to *plaque composition* and studied 15 patients with their first fatal acute myocardial infarction. The 4 major epicardial coronary arteries were sectioned at 5-mm intervals and a drawing of each of the resulting 555 Movat stained histologic sections was analyzed using a computerized morphometric system. The mean percent of dense relatively acellular fibrous tissue increased linearly from 15% in sections narrowed up to 25% in CSA to 55% in sections narrowed >75%. Cellular fibrous tissue containing variable amounts of elastic tissue decreased linearly from 83% in sections narrowed up to 25% in CSA to 12% in sections narrowed >75%. The mean percent occupied by densely calcified tissue increased linearly from 1% in sections narrowed <25% to 7% in sections narrowed >75% in CSA. The mean area occupied by pultaceous debris increased linearly from 0 to 16% over the range of CSA narrowing and from <25% narrowing to >75% narrowing. Thus, fibrous tissue is the dominant component of atherosclerotic plaques in patients with fatal first acute myocardial infarcts studied at necropsy.

Review of 18 publications before the wide-spread use of coronary care units disclosed that the frequency of *cardiac rupture* among necropsy cases of acute myocardial infarction range from 4 to 24% (mean 8%) (619 of 7,905 cases). We analyzed the frequency of cardiac rupture among patients studied at necropsy in the Pathology Branch since 1968 with fatal acute myocardial infarction. Of 648 such patients, 222 (34%) had cardiac rupture. Rupture occurred in 187 (43%) of 432 patients without healed myocardial infarcts and in 35 (16%) of 216 patients with a healed myocardial infarct. Thus, the frequency of cardiac rupture during acute myocardial infarction appears to have increased substantially since the wide-spread use of coronary care units, and the frequency of rupture is nearly 3 times greater in those whose rupture occurred during the first infarction compared to those with a previous infarct which healed.

We studied 138 patients with *rupture of the left ventricular free wall during acute myocardial infarction* (rupture group) and compared clinical and necropsy findings in them with 50 patients who died during their first myocardial infarction without rupture (non-rupture group). The frequency of systemic hypertension (55%-vs.-52%), angina pectoris (13%-vs.-22%) and congestive heart failure (0%-vs.-0%) before the fatal AMI was similar for both rupture and non-rupture groups. Mean heart weights for men (479 g-vs.-526 g) and women (399 g-vs.-432 g) with and without rupture also were insignificantly different. Left ventricular scar before the infarct which ruptured was present in 18 patients (13%); previous necropsy studies of fatal acute



myocardial infarction without rupture have indicated that about 50% have left ventricular scars. The rupture group had a significantly more frequent lateral wall location of the infarct (12%-vs.-2%). The number of 3 major (right, left anterior descending and left circumflex) epicardial coronary arteries narrowed at some point >75% in cross-sectional area by atherosclerotic plaque was significantly lower ( $p<.01$ ) in the rupture group (39%-vs.-58%). The percent of these 3 arteries totally occluded or nearly so (>95% in cross-sectional area) by plaque also was significantly less in the rupture group (24 of 198 arteries [12%] vs. 38 of 144 arteries [26%]). Analysis of each 5-mm long segment of these arteries in each group disclosed that the rupture group had significantly less narrowing than the non-rupture group. Of the 3287 five-mm segments of artery examined in the rupture group (66 patients), 512 (15%) were narrowed >75% in cross-sectional area by plaque; in contrast, of the 1848 five-mm segments in the non-rupture group (38 patients), 508 (28%) were narrowed to this degree by plaque ( $p<.0001$ ). Thus, rupture of the left ventricular free wall primarily is a complication of the first acute myocardial infarction and is associated with considerably less amounts of coronary narrowing than fatal acute myocardial infarction without rupture.

We studied 38 patients with *acquired ventricular septal defect during acute myocardial infarction* (rupture group) and compared clinical and necropsy findings in them with 50 patients who died during their first infarction without rupture (non-rupture group). The frequency of systemic hypertension (54%-vs.-52%), angina pectoris (28%-vs.-22%) and congestive heart failure (5 vs. 0%) before the fatal acute myocardial infarction was similar for both rupture and non-rupture groups. Mean heart weights for men (498 g.-vs.-526 g) and women (397 g.-vs.-432 g) with and without septal rupture also were insignificantly different. Only 4 (10%) of the rupture cases had a left ventricular scar before the infarct which ruptured, whereas previous studies of fatal acute myocardial infarction cases have shown that 50% of cases of fatal acute myocardial infarction without rupture have left ventricular scars. The rupture group had a significantly more frequent posterior location of the infarcts (74%-vs.-40%), and therefore, a higher frequency of associated right ventricular infarcts (50%-vs.-18%). The number of 3 major (right, left anterior descending and left circumflex) epicardial coronary arteries narrowed at some point >75% in cross-sectional area by atherosclerotic plaque was the same in both groups. The percent of these 3 arteries totally occluded or nearly so (>95% in cross-sectional area) by plaque was significantly less in the rupture group compared to the non-rupture group (9 of 99 arteries [9%]-vs.-38 of 144 arteries [26%]). Analysis of each 5-mm long segment of these arteries in each group disclosed that the rupture group had significantly less narrowing than the non-rupture group. Of the 825 five-mm segments of artery examined in the rupture group (18 patients), only 101 (13%) were narrowed >75% in cross-sectional area by plaque; in contrast, of the 1848 five-mm segments in the non-rupture group (38 patients), 508 (28%) were narrowed to this degree by plaque. Thus, rupture of the ventricular septum primarily is a complication of the first acute myocardial infarction and is associated with considerably less amounts of coronary narrowing than fatal acute myocardial infarction without rupture.



To determine the *relation between a single healed myocardial infarct to a fatal acute myocardial infarct* we studied 129 patients at necropsy with 1 grossly visible healed and 1 grossly visible acute infarct, and determined whether the acute infarct was opposite to or adjacent to the healed infarct or if 1 infarct was so large that it was both opposite to and adjacent to the other infarct. In 74 (57%) of the 129 patients, the 2 infarcts were opposite one another, in 40 (31%) they were adjacent, and in 15 (12%) they were both opposite and adjacent. The age, sex, mean size the healed infarct and heart weight was similar among the 3 groups.

To delineate their relation to outcome of *percutaneous transluminal coronary angioplasty* we determined *atherosclerotic plaque composition and coronary artery size* in 82 five-mm long segments at 28 PTCA sites in 26 patients having PTCA. The 26 patients were subdivided into 3 groups according to the degree of angiographic patency at the end of the PTCA procedure and to the duration of survival after PTCA ( $\leq 30$  or  $>30$  days): early success (13 patients, 16 PTCA sites and 49 five-mm segments); early failure (4 patients, 4 PTCA sites and 16 five-mm segments) and late success (9 patients, 8 PTCA sites and 17 five-mm segments). The mean percent of plaque comprised of fibrous tissue among the 3 groups was  $80 \pm 18\%$ ,  $71 \pm 23\%$ , and  $82 \pm 16\%$  (ns); the mean percent of plaque comprised of lipid was  $17 \pm 16\%$ ,  $21 \pm 24\%$ , and  $16 \pm 15\%$  (ns); and of calcium,  $3 \pm 4\%$ ,  $8 \pm 10\%$ , and  $2 \pm 3\%$  ( $p < 0.005$ ). The mean coronary arterial internal diameter (in mm) was  $3.3 \pm 0.6$ ,  $3.9 \pm 1.2$ , and  $3.2 \pm 0.7$  ( $p < 0.008$ ). Plaque tear was present in 1 or more histologic sections in 25 of the 26 patients and the 1 patient without it had the longest interval (nearly 3 years) between PTCA and death. Plaque tear extending from intima into media with dissection was observed only in the early and late success groups ( $p = 0.03$ ). Hemorrhage into plaque was present in 16 (80%) of 20 PTCA sites in the 2 early groups and in 3 (37%) of 8 sites in the late group ( $p < 0.03$ ). Occlusive thrombus (5 of 16, 1 of 4, and 1 of 8) and plaque debris (7 of 16, 1 of 4, and 2 of 8) in residual lumens were insignificantly different among the 3 groups and their 82 five-mm segments. Plaques that had  $>25\%$  lipid content, however, had an increased frequency of hemorrhage into plaque, occlusive thrombus and plaque debris in residual lumens. The findings in this study suggest that coronary arterial size and plaque composition are strong determinants of PTCA outcome. The ideal coronary arterial atherosclerotic narrowing for both technically and clinically successful PTCA appears to be a small ( $< 3.3$  mm in internal diameter) artery in which the plaque contains relatively little calcium and lipid.

Although available for patients undergoing coronary artery bypass grafting (CABG) for angina pectoris, necropsy data is lacking on patients undergoing *CABG shortly after an acute myocardial infarction*. We reviewed clinical and necropsy findings in 15 patients who underwent CABG within 1 month of onset of an acute myocardial infarction. All 15 patients had the acute myocardial infarction before admission to a hospital. The heart weights ranged from 375 to 690 g (mean 517). Fourteen hearts (93%) were of increased weight ( $>350$  g in women,  $>400$  g in men). Findings in the above 15 patients who had acute myocardial infarction before entering the cardiac catheterization laboratory and operating room (for CABG) and who survived  $<60$  days postoperatively were compared to those in 121 previously reported



necropsy patients who did not have an acute myocardial infarction (angina pectoris only) before CABG and who survived <60 days. Comparison between these 2 groups showed 1 important significant difference: the mean heart weight in the acute myocardial infarction group was significantly larger than that of the angina group (517 g vs. 444 g). Of the 15 patients, 14 (93%) in the acute myocardial infarction group had hearts of increased weight compared to 84 of 121 patients (69%) in the angina group.

#### MYOCARDIAL HEART DISEASE

Serial electrocardiographic changes in necropsy-proven idiopathic dilated cardiomyopathy are evaluated. In 34 patients with multiple electrocardiograms (mean 3/patient) progressive prolongation of PR interval ( $0.18 \pm 0.03$  to  $0.21 \pm 0.03$ ,  $p < 0.001$ ) and QRS duration ( $0.10 \pm 0.02$  to  $0.13 \pm 0.03$ ,  $p < 0.0001$ ) was noted. Progressive conduction abnormalities were common (82%). QTc interval, and QRS and T wave axes did not change. In 50 patients with electrocardiograms within 60 days of death, total 12-lead QRS and  $V_{1-6}$  QRS amplitude correlated with heart weight better than did the Estes-Romhilt score. The mean total 12-lead QRS amplitude was 138 mm with a mean of 106 for  $V_{1-6}$ . In 31 patients cardiac mass index was calculated and showed significant correlation with 12-lead and  $V_{1-6}$  QRS amplitudes. The QRS amplitudes remained constant during the illness. By utilizing total 12-lead QRS or frontal plane QRS amplitude heart weight can be predicted as early as 2 years before death. Utilization of body surface area and QRS amplitude criteria increases the accuracy of heart weight prediction. Thus, progressive electrocardiographic changes are common in patients with idiopathic dilated cardiomyopathy and QRS amplitude criteria are more accurate in the prediction of left ventricular hypertrophy than standard criteria.

#### CONGENITAL HEART DISEASE

*Anomalous origin of either the left main coronary artery (LMCA) or right coronary artery (RCA) from the aorta with subsequent coursing between the aorta and pulmonary trunk is a rare and sometimes fatal coronary anomaly.* We reviewed 32 cases of these anomalies with particular attention to the exact location and shape of the anomalistically positioned ostium and coronary dominance. The LMCA (7 cases) arose either from behind the right coronary sinus (6 cases) or a single ostium with the RCA straddling the right-left commissure and right coronary sinus (1 case). In 5 of the 7 cases, the anomaly was fatal. In 6 cases of anomalous origin of the LMCA, the RCA was dominant and in 4 the anomaly was fatal. In only 1 case of anomalous origin of the LMCA, the left circumflex coronary artery was dominant and in this case the anomaly also was fatal. The RCA (25 cases) arose either from behind the left coronary sinus (8 cases), above the left coronary sinus (5 cases), from above the right-left commissure (10 cases), or as a single ostium with the LMCA above the right-left commissure and left coronary sinus (2 cases). In 8 of these 25 cases the anomaly was fatal. In 7 cases of anomalous origin of the RCA, the left circumflex coronary artery was dominant and in 1 the anomaly was clinically significant. In 1 case, both the RCA and left circumflex coronary artery were hypoplastic and the anomaly was fatal. Coronary dominance, not ostial shape, was useful in separating the clinically significant from the clinically insignificant anomalies.





*Congenitally quadricuspid aortic valves* are rare and usually cause no valvular dysfunction. We studied 6 patients with quadricuspid aortic valves. One quadricuspid aortic valve was severely regurgitant, necessitating surgical replacement. The 5 remaining valves functioned normally and each of these 5 patients died from a non-cardiac condition. Other congenital cardiac abnormalities were present in 2 of the 6 patients. The accessory cusp in all 6 patients was smaller than any of the other 3 cusps which were similar in size. The accessory cusp was between the right and posterior cusps in 4 patients, between the left and posterior cusps in case 1, and between the right and left cusps in case 2.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03922-01 PA

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Congenitally Quadricuspid Aortic Valve: Analysis of 6 Necropsy Patients

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: William C. Roberts, MD

Others:

Daniel J. Fernicola, MD\*

Jessica M. Mann, MD

## COOPERATING UNITS (if any)

\*Intern, Department of Medicine, Thomas Jefferson Hospital, Philadelphia, Pennsylvania 19107.

## LAB/BRANCH

Pathology Branch

## SECTION

Cardiac Pathology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

0.03

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Congenitally quadricuspid aortic valves are rare and usually cause no valvular dysfunction. We studied 6 patients with quadricuspid aortic valves. One quadricuspid aortic valve was severely regurgitant, necessitating surgical replacement. The 5 remaining valves functioned normally and each of these 5 patients died from a non-cardiac condition. Other congenital cardiac abnormalities were present in 2 of the 6 patients: case 1 had a "ventricular septal defect" which had closed spontaneously and case 2 had a congenitally deficient posterior one half of the anterior mitral leaflet and no attached chordae tendineae to it. The accessory cusp in all 6 patients was smaller than any of the other 3 cusps which were similar in size. The accessory cusp was between the right and posterior cusps in 4 patients, between the left and posterior cusps in case 1, and between the right and left cusps in case 2. The right coronary artery was dominant in the 5 patients in whom coronary dominance was known.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03923-01 PA

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Location of an AMI in patients with a healed infarct

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

William C. Roberts, MD

Others:

Benjamin N. Potkin, MD, Division of Cardiology, Department of Medicine,  
Cedars-Sinai Medical Center, Los Angeles, CA 90048

## COOPERATING UNITS (if any)

Cedars-Sinai Medical Center, Los Angeles, CA 90048

## LAB/BRANCH

Pathology Branch, NHLBI

## SECTION

Cardiac Pathology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

0.02

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

To determine the relation of a single healed myocardial infarct to a fatal acute myocardial infarct we studied 129 patients at necropsy with 1 grossly visible healed and 1 grossly visible acute infarct, and determined whether the acute infarct was opposite to or adjacent to the healed infarct or if one infarct was so large that it was both opposite to and adjacent to the other infarct. In 74 (57%) of the 129 patients, the 2 infarcts were opposite one another, in 40 (31%) they were adjacent, and in 15 (12%) they were both opposite and adjacent. The age, sex, mean size of the healed infarct and heart weight were similar among the 3 groups. Acute myocardial infarcts were larger in the group that had both opposite and adjacent infarcts than either of the other 2 groups ( $p < 0.001$ ). Information regarding whether the infarcts were clinically recognized or not was available in 108 patients: both infarcts were recognized in 41 (38%), neither infarct was recognized in 15 (14%), and 1 infarct was recognized and the other was not in 52 (48%). The number of the 4 major epicardial coronary arteries narrowed at some point  $> 75\%$  in cross-sectional area by atherosclerotic plaque was similar in patients with recognized and in those with unrecognized infarcts. Similar numbers of narrowed major epicardial coronary arteries also were found in each of the 3 infarct groups (opposite, adjacent or both).

694



## NOTICE OF INTRAMURAL RESEARCH PROJECT

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Effects of PTCA on atherosclerotic plaques and relation of plaque composition

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: William C. Roberts, MD

Others:

Benjamin N. Potkin, MD

## COOPERATING UNITS (if any)

Division of Cardiology, Department of Medicine, Cedars-Sinai Medical Center,  
Los Angeles, CA 90048

## LAB/BRANCH

Pathology Branch

## SECTION

Cardiac Pathology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

0.02

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Plaque tear extending from intima into media with dissection was observed only in the early and late success groups ( $p=0.03$ ). Hemorrhage into plaque was present in 16 (80%) of 20 PTCA sites in the 2 early groups and in 3 (37%) of 8 sites in the late group ( $p<0.03$ ). Occlusive thrombus (5 of 16, 1 of 4, and 1 of 8) and plaque debris (7 of 16, 1 of 4, and 2 of 8) in residual lumens were insignificantly different among the 3 groups and their 82 five-mm segments. Plaques that had  $>25\%$  lipid content, however, had an increased frequency of hemorrhage into plaque ( $p<0.004$ ), occlusive thrombus ( $p=0.0001$ ) and plaque debris in residual lumens ( $p<0.05$ ). The findings in this study suggest that coronary arterial size and plaque composition are strong determinants of PTCA outcome. The ideal coronary arterial atherosclerotic narrowing for both technically and clinically successful PTCA appears to be a small ( $<3.3$  mm in internal diameter) artery in which the plaque contains relatively little calcium and lipid.

696





## NOTICE OF INTRAMURAL RESEARCH PROJECT

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Acquired Ventricular Septal Defect During Acute Myocardial Infarction

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: William C. Roberts, MD, Chief, Pathology Branch, NHLBI

Others:

Jessica M. Mann, MD, Senior Staff Fellow, Pathology Branch, NHLBI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Pathology Branch, NHLBI

## SECTION

Cardiac Pathology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

0.02

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects☐ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

We studied 38 patients (24 men and 14 women) with an acquired ventricular septal defect during acute myocardial infarction (AMI) (rupture group) and compared clinical and necropsy findings in them with 50 patients who died during their first AMI without rupture (non-rupture group). The frequency of systemic hypertension (54%-vs.-52%), angina pectoris (28%-vs.-22%) and congestive heart failure (5%-vs.-0%) before the fatal AMI was similar for both rupture and non-rupture groups. Mean heart weights for men (498 g-vs.-526 g) and women (397 g-vs.-432 g) with and without septal rupture also were insignificantly different. Whereas previous studies of fatal AMI cases have shown that 50% of cases of fatal AMI without rupture have left ventricular scars, only 4 (10%) of the rupture cases had a left ventricular scar before the infarct which ruptured. The rupture group had a significantly more frequent ( $p < 0.01$ ) posterior location of the infarcts (74%-vs.-40%), and therefore, a higher frequency of associated right ventricular infarcts 50%-vs.-18%). The number of 3 major (right, left anterior descending and left circumflex) epicardial coronary arteries narrowed at some point  $> 75\%$  in cross-sectional area by atherosclerotic plaque was the same in both groups. The percent of these 3 arteries totally occluded or nearly so ( $> 95\%$  in cross-sectional area) by plaque was significantly less ( $p < .001$ ) in the rupture group compared to the non-rupture group (9 of 99 arteries [9%]-vs.-38 of 144 arteries [26%]). Analysis of each 5-mm long segment of these arteries in each group disclosed that the rupture group had significantly less narrowing than the non-rupture group.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03926-01 PA

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Rupture of the Left Ventricular Free Wall During Acute Myocardial Infarction:

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: William C. Roberts, MD

Others:

Jessica M. Mann, MD

## COOPERATING UNITS (if any)

## LAB/BRANCH

Pathology Branch, NHLBI

## SECTION

Cardiac Pathology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

0.02

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects☒ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We studied 138 patients (69 men and 69 women) with rupture of the left ventricular (LV) free wall during acute myocardial infarction (AMI) (rupture group) and compared clinical and necropsy findings in them with 50 patients who died during their first AMI without rupture (non-rupture group). The frequency of systemic hypertension (55%-vs.-52%), angina pectoris (13%-vs.-22%) and congestive heart failure (0%-vs.-0%) before the fatal AMI was similar for both rupture and non-rupture groups. Mean heart weights for men (479 g-vs.-526 g) and women (399 g-vs.-432 g) with and without rupture also were insignificantly different. LV scar before the infarct which ruptured was present in 18 patients (13%); previous necropsy studies of fatal AMI without rupture have indicated that 50% have LV scars. The rupture group had a significantly more frequent ( $p<.01$ ) lateral wall location of the infarct (12%-vs.-2%). The number of 3 major (right, left anterior descending and left circumflex) epicardial coronary arteries narrowed at some point  $>75\%$  in cross-sectional area by atherosclerotic plaque was significantly lower ( $p<.01$ ) in the rupture group (39%-vs.-58%). The percent of these 3 arteries totally occluded or nearly so ( $>95\%$  in cross-sectional area) by plaque also was significantly less ( $p<.001$ ) in the rupture group (24 of 198 arteries [12%]-vs.-38 of 144 arteries [26%]). Analysis of each 5-mm long segment of these arteries in each group disclosed that the rupture group had significantly less narrowing than the non-rupture group.

700



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03927-01 PA

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Serial Electrocardiographic Changes in Idiopathic Dilated Cardiomyopathy

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: William C. Roberts, MD

Others:

Robert L. Wilensky, MD\*

Paul Yudelman, MD\*

Andrew I. Cohen, MD\*

Ross D. Fletcher, MD\*

James Atkinson, MD\*

Renu Virmani, MD\*

COOPERATING UNITS (If any)

\*Pls. see attached sheet.

LAB/BRANCH

Pathology Branch

SECTION

Cardiac Pathology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

1.0

PROFESSIONAL:

0.07

OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects

☒ (b) Human tissues

☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Serial electrocardiographic changes in necropsy-proven idiopathic dilated cardiomyopathy are evaluated. In addition, a method of predicting heart weight, using QRS amplitudes is described. In 34 patients with multiple electrocardiograms (mean 3/patient) progressive prolongation of PR interval ( $0.18 \pm 0.03$  to  $0.21 \pm 0.03$ ,  $p < 0.001$ ) and QRS duration ( $0.10 \pm 0.02$  to  $0.13 \pm 0.03$ ,  $p < 0.0001$ ) was noted. Progressive conduction abnormalities were common (82%). QTc interval, and QRS and T wave axes did not change. In 50 patients with electrocardiograms within 60 days of death total 12-lead QRS and  $V_{1-6}$  QRS amplitude correlated with heart weight ( $r = 0.51$ ,  $p < 0.0001$  and  $r = 0.55$ ,  $p < 0.0001$ ) better than did the Estes-Romhilt score. The mean total 12-lead QRS amplitude was 138 mm with a mean of 106 for  $V_{1-6}$ . In 31 patients cardiac mass index was calculated and showed significant correlation with 12-lead and  $V_{1-6}$  QRS amplitudes ( $r = 0.68$ ,  $p < 0.0001$  and  $r = 0.75$ ,  $p < 0.0001$ , respectively). The QRS amplitudes remained constant during the illness. By utilizing total 12-lead QRS or frontal plane QRS amplitude heart weight can be predicted as early as 2 years prior to death. Utilization of body surface area and QRS amplitude criteria increases the accuracy of heart weight prediction. Thus, we conclude that progressive electrocardiographic changes are common in patients with idiopathic dilated cardiomyopathy and that QRS amplitude criteria are more accurate in the prediction of left ventricular hypertrophy than standard criteria.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03928-01 PA

## PERIOD COVERED

October 1, 1987 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Anomalous Origin of Either Right or Left Main Coronary Artery From The Aorta

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: William C. Roberts, MD

Others:

Amy H. Kragel, MD

## COOPERATING UNITS (if any)

## LAB/BRANCH

Pathology Branch, NHLBI

## SECTION

Cardiac Pathology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

0.02

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Anomalous origin of either the left main coronary artery (LMCA) or right coronary artery (RCA) from the aorta with subsequent coursing between the aorta and pulmonary trunk is a rare and sometimes fatal coronary artery anomaly. We reviewed 32 cases of these anomalies with particular attention to the exact location and shape of the anomalistically positioned ostium and coronary dominance. The LMCA (7 cases) arose either from behind the right coronary sinus (6 cases) or as a single ostium with the RCA straddling the right-left commissure and right coronary sinus (1 case). In 5 of the 7 cases, the anomaly was fatal. In 6 cases of anomalous origin of the LMCA, the RCA was dominant and in 4 the anomaly was fatal. In only 1 case of anomalous origin of the LMCA, the left circumflex coronary artery was dominant and in this case the anomaly also was fatal. The RCA (25 cases) arose either from behind the left coronary sinus (8 cases), above the left coronary sinus (5 cases), from above the right-left commissure (10 cases), or as a single ostium with the LMCA above the right-left commissure and left coronary sinus (2 cases). In 8 of these 25 cases the anomaly was fatal. In 7 cases of anomalous origin of the RCA, the left circumflex coronary artery was dominant and in 1 the anomaly was clinically significant. In 1 case, both the RCA and left circumflex coronary artery were hypoplastic and the anomaly was fatal. Coronary dominance, not ostial shape, was useful in separating the clinically significant from the clinically insignificant anomalies.

705





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03929-01 PA

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Morphometric analysis of the composition of atherosclerotic plaques

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: William C. Roberts, MD

Others:

Amy H. Kragel, MD

Janet T. Wittes, PhD.\*

## COOPERATING UNITS (if any)

\*Biostatistics Research Branch, NHLBI, Bethesda, MD

## LAB/BRANCH

Pathology Branch

## SECTION

Cardiac Pathology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

0.02

## OTHER:

0.01

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☒ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Coronary artery plaque composition was studied in 15 patients with fatal first acute myocardial infarction. The 4 major (left main, left anterior descending, left circumflex, and right) epicardial coronary arteries were sectioned at 5-mm intervals and a drawing of each of the resulting 555 Movat-stained histologic sections was analyzed using a computerized morphometry system. The mean percent of dense relatively acellular fibrous tissue increased linearly from 15% in sections narrowed up to 25% in cross-sectional area (CSA) to 55% in sections narrowed >75% in CSA ( $p < 0.0001$ ). Cellular fibrous tissue containing variable amounts of elastic tissue decreased linearly from 83% in sections narrowed up to 25% in CSA to 12% in sections narrowed >75% in CSA ( $p < 0.0001$ ). The mean percent occupied by densely calcified tissue increased linearly from 1% in sections narrowed <25% in CSA to 7% in sections narrowed >75% in CSA ( $p < 0.0001$ ). The mean area occupied by pultaceous debris increased linearly from 0 to 16% over the range of CSA narrowing from <25% CSA narrowing to >75% CSA narrowing. Statistically significant differences in percent of plaque containing pultaceous debris and cellular fibrous tissue were found between sections narrowed 51-75% and 76-100% in CSA. Pultaceous debris increased from 6% to 16% ( $p < 0.0001$ ) and cellular fibrous tissue fell from 23% to 12% ( $p = 0.04$ ). Thus, the dominant component of atherosclerotic plaques in the major epicardial coronary arteries of patients with fatal acute myocardial infarction is fibrous tissue. With increasing degrees of CSA narrowing, the percent of cellular fibrous tissue decreases, and the percent of acellular fibrous tissue, calcific deposits, pultaceous debris (extracellular lipid) and inflammatory infiltrates increase.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03930-01 PA

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Morphologic Findings in Patients Undergoing Coronary Artery Bypass Grafting

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: William C. Roberts, MD

Others:

Jay M. Kalan, MD

## COOPERATING UNITS (if any)

## LAB/BRANCH

Pathology Branch

## SECTION

Cardiac Pathology Branch

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

0.02

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Although available for patients undergoing coronary artery bypass grafting (CABG) for angina pectoris (1-3), necropsy data is lacking on patients undergoing CABG shortly after an acute myocardial infarction (AMI). Herein, we describe certain clinical and necropsy findings in 15 patients who underwent CABG within 1 month of onset of an AMI. The records of all patients having had CABG and studied subsequently at the Pathology Branch, National Heart, Lung, and Blood Institute, were reviewed. Fifteen patients had CABG without simultaneous left ventricular aneurysmectomy or cardiac valve replacement within 30 days of onset of AMI and they form the basis of this study. All 15 patients had the AMI before admission to a hospital. The heart weights ranged from 375 to 690 g (mean 517). Fourteen hearts (93%) were of increased weight (>350 g in women, >400 g in men). Findings in the above 15 patients who had AMI before entering the cardiac catheterization laboratory and operating room (for CABG) and who survived <60 days postoperatively were compared to those in 121 previously reported (3) necropsy patients who did not have an AMI (angina pectoris only) before CABG and who survived <60 days (Table II). Comparison between these 2 groups showed 1 important significant difference: the mean heart weight in the AMI group was significantly larger than that of the angina group (517 g vs 444 g). Of the 15 patients, 14 (93%) in the AMI group had hearts of increased weight compared to 84 of 121 patients (69%) in the angina group ( $p < .05$ ).



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03931-01 PA

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Sudden death & cardiomegaly unassociated with coronary artery disease

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: William C. Roberts, MD

Others:

Amy H. Kragel, MD

## COOPERATING UNITS (if any)

## LAB/BRANCH

Pathology Branch

## SECTION

Cardiac Pathology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

0.02

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☒ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Sudden death has many causes and necropsy usually allows delineation of the specific cause. During the past 8 years we have studied at necropsy 6 patients, all man, who died suddenly outside the hospital and although necropsy disclosed cardiomegaly, a specific cause of death was not discernible. Certain clinical and morphologic features in these 6 patients are described. All 6 patients died outside the hospital. The most likely cause for the cardiomegaly in our 6 patients is systemic hypertension. Systemic arterial pressure was available in only 3 of the 6 patients and in each it was stated to have been elevated. None of the 6 patients had narrowing of any epicardial coronary artery, none had anatomic evidence of valvular, myocardial, congenital or pericardial heart disease. None had a coronary arterial anomaly. Cases of morbid obesity (>250 pounds) were excluded from this study. Although it is well recognized that patients with systemic hypertension have an increased frequency of both atrial and ventricular arrhythmias compared with normotensive persons and that hypertensive persons with left ventricular hypertrophy have a higher frequency of these arrhythmias compared with hypertensive persons without left ventricular hypertrophy, the occurrence of sudden death in persons with systemic hypertension in the absence of significant narrowing of 1 or more epicardial coronary arteries has not been described previously to our knowledge.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03932-01 PA

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Evidence that Elevation of the Blood Non-High Density Lipoprotein

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: William C. Roberts, MD

## COOPERATING UNITS (if any)

## LAB/BRANCH

Pathology Branch

## SECTION

Cardiac Pathology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

0.01

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Although the relation between cholesterol and atherosclerosis has been discussed for nearly 80 years, only in recent years has sufficient evidence accumulated to indicate without reasonable doubt that cholesterol plays a major role in the development of atherosclerotic plaques, and, therefore, symptomatic and fatal atherosclerotic coronary artery disease. This piece reviews the various factors linking cholesterol to atherosclerosis: 1) Feeding high cholesterol diets to certain non-human animals produces atherosclerotic plaques similar to those occurring in humans. 2) Cholesterol is found in both experimentally induced atherosclerotic plaques in non-human animals and in plaques in humans. 3) Atherosclerotic plaques large enough to produce clinical problems occur only in persons having serum or plasma total cholesterol levels for long periods of time  $>150$  mg/dl. 4) The higher the blood total cholesterol level the greater the chance of having symptomatic and fatal atherosclerotic disease. 5) The higher the serum total and LDL cholesterol levels the greater the extent of the atherosclerotic plaques. 6) Lowering the blood total cholesterol and LDL cholesterol levels decreases the chances of fatal or non-fatal atherosclerotic disease. 7) Atherosclerotic plaques regress when high blood cholesterol levels are lowered.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03933-01 PA

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Frequency of Acute and Healed Myocardial Infarcts in Fatal Cardiac Amyloidosis

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: William C. Roberts, MD

Others:

Deborah J. Barbour, MD

## COOPERATING UNITS (if any)

## LAB/BRANCH

Pathology Branch

## SECTION

Cardiac Pathology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

0.02

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Transmural (involvement of more than the inner one-half of the myocardial wall) left ventricular necrosis or fibrosis is most often secondary to severe (>75% cross-sectional area) narrowing by atherosclerotic plaques of 1 or more of the major epicardial coronary arteries. Myocardial infarction also may occur without significant narrowing of an epicardial coronary artery in several conditions, including hypertrophic cardiomyopathy, dilated cardiomyopathy, left or right ventricular outflow obstruction, and anomalies of coronary arterial origin or course. Another condition associated with myocardial infarction without significant narrowing of 1 or more of the epicardial coronary arteries is cardiac amyloidosis. Of 61 necropsy patients aged 21 to 97 years (mean 64) with cardiac amyloidosis severe enough to cause cardiac dysfunction studied in this laboratory during the past 28 years, 3 (5%) had transmural necrosis and 5 (8%) had transmural fibrosis of the left ventricular wall, and 53 (87%) had neither necrosis nor fibrosis. One patient with necrosis, 3 with fibrosis, and 9 with neither necrosis nor fibrosis and 1 or more epicardial coronary arteries severely narrowed by atherosclerotic plaque. Two patients with necrosis and 2 with fibrosis, plus 44 with neither, had no epicardial coronary artery narrowed >75% in cross-sectional area by atherosclerotic plaque.

715



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03934-01 PA

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Frequency of cardiac rupture (LVFW, ventricular septum or papillary muscle)

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: William C. Roberts, MD

Others:

Shanthasundari G. Reddy, MD\*

## COOPERATING UNITS (if any)

\*Visiting Associate, NHLBI, Pathology Branch, Bethesda, MD 20892

## LAB/BRANCH

Pathology Branch

## SECTION

Cardiac Pathology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

0.01

## OTHER:

0.01

## CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects☒ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Review of 18 publications before the widespread use of cardiac care units disclosed that the frequency of cardiac rupture among necropsy cases of acute myocardial ranged from 4 to 24% (mean 8%) (619 of 7905 cases). We analyzed the frequency of cardiac rupture among patients studied at necropsy in our laboratory since 1968 fatal acute myocardial infarction. Of 648 such patients, 222 (34%) had cardiac rupture (left ventricular free wall, ventricular septum and/or papillary muscle). Rupture occurred in 187 (43%) of 432 patients without healed myocardial infarcts (grossly visible left ventricular scars), and in 35 (16%) of 216 patients with a healed myocardial infarct ( $p < .01$ ). Thus, the frequency of cardiac rupture during acute myocardial infarction appears to have increased substantially since the widespread use of coronary care units, and the frequency of rupture is nearly 3 times greater in those in whom rupture occurred during the first acute myocardial infarction compared to those with a previous infarct which healed.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 03935-01 PA

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Minoxidil-induced cardiotoxicity in miniature swine.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI

Others:

Eugene H. Herman, Division of Drug Biology, FDA, Washington, DC

Robert S. K. Young, Division of Drug Biology, FDA, Washington, DC

T. Balazs, Division of Drug Biology, FDA, Washington, DC

## COOPERATING UNITS (if any)

FDA, Washington, DC

## LAB/BRANCH

Pathology Branch

## SECTION

Ultrastructure Section

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

0.05

## PROFESSIONAL:

0.05

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Oral administration of minoxidil, a vasodilating antihypertensive agent, in doses of 1, 3 or 10 mg/kg, on 2 consecutive days, produced atrial and ventricular lesions in miniature swine. The atrial lesions were characterized by a hemorrhagic arteritis, and the ventricular lesions consisted of focal areas of left ventricular papillary muscle necrosis.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03936-01 PA

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Pulmonary elastic fiber degradation in paraquat toxicity.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Víctor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI

Others: Yuh Fukuda, Visiting Scientist, Pathology Branch, NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Pathology Branch

## SECTION

Ultrastructure Section

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

0.05

## PROFESSIONAL:

0.05

## OTHER:

-

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Peroxidase-labeled and ferritin-labeled antibodies were used for the light and electron microscopic localization of elastin in lungs of monkeys with paraquat toxicity.

721





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03937-01 PA

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

MM-159 protects against doxorubicin-induced chronic cardiotoxicity.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI

Others: Eugene H. Herman, Division of Drug Biology, FDA, Washington, DC

H.B. Bhat, College of Pharmacy, Ohio State University, Columbus, Ohio

D.T. Witiak, College of Pharmacy, Ohio State University, Columbus, Ohio

## COOPERATING UNITS (if any)

FDA

College of Pharmacy, Ohio State University

## LAB/BRANCH

Pathology Branch

## SECTION

Ultrastructure Section

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

0.05

## PROFESSIONAL:

0.05

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

MM-159 (bis-4-morpholinomethyl-3,5-dioxopiperazinyl-1,2-propane), a new derivative of ICRF-187, was found to have marked cardioprotective activity against the chronic cardiotoxicity produced by doxorubicin, a highly effective antineoplastic agent.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03938-01 PA

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Polysaccharide storage disease with severe cardiac involvement.

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI  
Others: GM Greene, DC Weldon, JP Cheatham, RD McComb, BI Brown, CH Gumbiner, JA Vanderhoof, PG Itkin, and BM McManus, Departments of Medicine and Pathology, University of Nebraska Medical Center, Omaha, Nebraska.

COOPERATING UNITS (if any)

University of Nebraska Medical Center

LAB/BRANCH

Pathology Branch

SECTION

Ultrastructure Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

TOTAL MAN-YEARS:

0.05

PROFESSIONAL:

0.05

OTHER:

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects

☒ (b) Human tissues

☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A description is made of clinical, biochemical and morphological findings in a patient with polysaccharide storage disease, a poorly understood disorder which seems to be related to type IV glycogen storage disease.

725



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03939-01 PA

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Chloroquine-induced cardiomyopathy

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI  
Others:H. A. McAllister, R. J. Hall, N. E. Strickman and M. Bossart, Departments of  
Medicine and Pathology, Saint Luke's Episcopal Hospital, Houston, Texas.

## COOPERATING UNITS (if any)

Saint Luke's Episcopal Hospital, Houston, Texas.

## LAB/BRANCH

Pathology Branch

## SECTION

Ultrastructure Section

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

0.05

## PROFESSIONAL:

0.05

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Accumulations of electron-dense concentric and parallel lamellae and curvilinear bodies within cardiac myocytes were found in two patients in whom biventricular failure developed during treatment of systemic lupus erythematosus with chloroquine.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03940-01 PA

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Morphology of the heart in left ventricular hypertrophy.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI

Others:

Rene Rodriguez, Visiting Fellow, Pathology Branch, NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Pathology Branch

## SECTION

Ultrastructure Section

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

0.05

## PROFESSIONAL:

0.05

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A review is presented of the morphological alterations that occur in the heart in left ventricular hypertrophy.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03941-01 PA

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Doxorubicin-induced cardiotoxicity in spontaneously hypertensive rats.

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI

Others:

Eugene H. Herman, Division of Drug Biology, FDA, Washington, DC

Antione El-Hage, Division of Drug Biology, FDA, Washington, DC

COOPERATING UNITS (if any)

FDA

LAB/BRANCH

Pathology Branch

SECTION

Ultrastructure Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

TOTAL MAN-YEARS:

0.05

PROFESSIONAL:

0.05

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Spontaneously hypertensive rats were found to be much more sensitive than normotensive Wistar-Kyoto rats to the cardiotoxic and nephrotoxic effects of doxorubicin.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03942-01 PA

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Evaluation of explanted polyurethane valves.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI

Others: Stephen L. Hilbert, Center for Devices and Radiological Health,  
FDA, Washington, DC

Y. Tomita, Visiting Fellow, Surgery Branch, NHLBI

E. E. Eidbo, Biological Technician, Surgery Branch, NHLBI

Michael Jones, Senior Surgeon, Surgery Branch, NHLBI

## COOPERATING UNITS (if any)

FDA

Surgery Branch, NHLBI

## LAB/BRANCH

Pathology Branch

## SECTION

Ultrastructure Section

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

0.05

## PROFESSIONAL:

0.05

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Morphologic, chemical and hemodynamic studies were made of polyurethane trileaflet cardiac valve prostheses that had been implanted in young sheep for 17 to 21 weeks in the mitral position. These valves were found to have become both stenotic and regurgitant and to have developed calcific deposits.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03943-01 PA

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

ICRF-187 protects against the cardiotoxicity induced by large doses of doxorubicin.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI  
Others:

Eugene H. Herman, Division of Drug Biology, FDA, Washington, DC

Robert L. Hamlin, College of Pharmacy, Ohio State University, Columbus, Ohio

## COOPERATING UNITS (if any)

FDA

Ohio State University

## LAB/BRANCH

Pathology Branch

## SECTION

Ultrastructure Section

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

0.1

## PROFESSIONAL:

0.1

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A study in beagle dogs showed that the administration of ICRF-187 provided a marked protective effect against the chronic cardiotoxicity produced by doxorubicin, and permitted the administration of very large doses of this antineoplastic agent (four times the usual dose).



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03944-01 PA

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Animal model of acute and chronic pericarditis.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI

Others:

Lee V. Leak, Department of Anatomy, Howard University College of Medicine,  
Washington, DC

Stephen E. Cohen, Clinical Associate, Surgery Branch, NHLBI

Michael Jones, Senior Surgeon, Surgery Branch, NHLBI

## COOPERATING UNITS (if any)

Howard University College of Medicine  
Surgery Branch, NHLBI

## LAB/BRANCH

Pathology Branch

## SECTION

Ultrastructure Section

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

0.05

## PROFESSIONAL:

0.05

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

To study the histologic and ultrastructural changes occurring in pericardial inflammation, a model of pericarditis was developed in sheep by surgically injecting heat-killed staphylococci and Freund's adjuvant into the pericardial cavity under sterile conditions.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03945-01 PA

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Myocyte hypertrophy vs. hyperplasia in hypertrophic cardiomyopathy.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI

Others:

Rene Rodriguez, Visiting Fellow, Pathology Branch, NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Pathology Branch

## SECTION

Ultrastructure Section

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

0.5

## PROFESSIONAL:

0.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects☒ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Evidence is presented to show that the increase in cardiac mass which occurs in hypertrophic cardiomyopathy is mediated at least in part by hyperplasia rather than by hypertrophy of the myocytes.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03946-01 PA

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Sarcoplasmic reticulum structure in the conduction system of the heart.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI  
Others: Y. Tomita, Visiting Fellow, Surgery Branch, NHLBI.

## COOPERATING UNITS (if any)

Surgery Branch, NHLBI

## LAB/BRANCH

Pathology Branch

## SECTION

Ultrastructure Section

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

0.05

## PROFESSIONAL:

0.05

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The osmium-ferrocyanide method for staining of the sarcoplasmic reticulum was used for a morphological study of the various components of the sarcoplasmic reticulum in the atrioventricular node and bundle cells of guinea pig hearts. The results obtained show that each of the regions of the node and bundle have a different, characteristic pattern of distribution of components of sarcoplasmic reticulum.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03947-01 PA

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Lamellar structures in lavage fluid in pulmonary alveolar proteinosis.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI

Others: T. Takemura, Visiting Scientist, Pathology Branch, NHLBI

Y. Fukuda, Visiting Scientist, Pathology Branch, NHLBI

M. Harrison, Electron microscopy technician, Clinical Hematology Branch, NHLBI.

## COOPERATING UNITS (if any)

Clinical Hematology Branch, NHLBI

## LAB/BRANCH

Pathology Branch

## SECTION

Ultrastructure Section

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

0.05

## PROFESSIONAL:

0.05

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Ultrastructural, histochemical and freeze-fracture studies of material recovered by bronchoalveolar lavage from patients with pulmonary alveolar proteinosis revealed four distinct types of multilamellated structures. Demonstration of these structures is important in establishing the diagnosis of pulmonary alveolar proteinosis.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03948-01 PA

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Use of ferrocyanide-osmium stain to demonstrate the structure of the T ssytem.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI

Others: Y. Tomita, Visiting Fellow, Surgery Branch, NHLBI

## COOPERATING UNITS (if any)

Surgery Branch, NHLBI

## LAB/BRANCH

Pathology Branch

## SECTION

Ultrastructure Section

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

0.05

## PROFESSIONAL:

0.05

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The structure of the transverse tubular system in ventricular and atrial myocytes of the rat was investigated by examining sections stained with the osmium-ferrocyanide method. Ventricular myocytes contained longitudinally and transversely oriented tubules which represent invaginations of the plasma membranes and form orderly latticeworks. In atrial myocardium these structures are poorly developed and more pleomorphic. This stain reveals the transverse tubular system to be more extensive and complex than is evident from examination of routinely stained preparations.

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Annual Report of the Pulmonary Branch  
National Heart, Lung, and Blood Institute  
October 1, 1987 through September 30, 1988

The research of the Pulmonary Branch centers on diseases of the alveolar structures, the site in the body where gases are exchanged between air and blood. All of the disorders under investigation are chronic, progressive, and often fatal. Together they affect more than two million individuals in the USA. Three categories of disease are investigated: destructive disorders, fibrotic disorders, and granulomatous disorders. All are characterized by chronic inflammatory processes in the lower respiratory tract. This local inflammation is central to the pathogenesis of each disorder as it is the inflammation that causes the changes in the lung parenchyma that results in lung dysfunction and eventual failure of the lung as an organ of gas exchange. In this context, the research of the Pulmonary Branch utilizes the tools of cellular biology, immunology, and molecular biology to understand the pathogenesis of these disorders and to develop new therapeutic strategies to prevent the progressive loss of functioning alveolar-capillary units.

Inflammation in the lower respiratory tract can be evaluated by bronchoalveolar lavage, a technique that permits direct access to the epithelial surface of the lower respiratory tract where inflammatory cells and relevant molecules in the epithelial lining fluid can be easily and repetitively sampled. To accomplish bronchoalveolar lavage, local anesthesia is administered to the upper respiratory tract and a fiberoptic bronchoscope is gently wedged into a distal bronchus. Sterile saline is infused into the bronchoscope and then suctioned back, thus recovering lower respiratory tract epithelial lining fluid and its contents. Over the past decade, this procedure has been used by the Pulmonary Branch to evaluate several thousand individuals. Using available cellular and molecular biologic methods, the numbers of inflammatory cells recovered by this procedure are sufficient to evaluate the in situ expression of the host of genes used by inflammatory cells participating in the pathogenesis of these disorders.

In addition to direct evaluation of the inflammatory processes ongoing in the lower respiratory tract, a significant amount of work by the Pulmonary Branch has been directed toward the structure and expression of genes coding for molecules that are capable of injuring the lung or protecting the lung from such injury. These studies relate to both basic mechanisms and hereditary disorders in which mutations in such genes have a profound influence on susceptibility to lung disease.

#### I. Disorders characterized by destruction of the alveolar walls.

This group of disorders, referred to as "emphysema," is associated with progressive dissolution of the lung parenchyma. The destruction of the alveolar walls is caused by a combination of events: a chronic, mild inflammatory process dominated by alveolar macrophages with smaller numbers of neutrophils and a faulty protective screen insufficient to defend the alveolar walls from proteolytic enzymes released by the inflammatory cells.

The most important of these proteolytic enzymes is neutrophil elastase (NE), a 218 amino acid single chain glycoprotein that functions as a potent pro-



teolytic enzyme capable of destroying a broad range of substrates in the physiologic conditions of the extracellular milieu. There are at least three forms of NE; current evidence suggests all have the same polypeptide backbone but differ in the two asparaginyl N-linked carbohydrate side-chains. The protein contains four intramolecular disulfide links and is very rich in arginyl residues, located mostly on the outside of the molecule, giving it a very high isoelectric point. NE is classified as a "serine protease"; its reactive site is comprised of the "catalytic triad" His<sup>41</sup>--Asp<sup>88</sup>--Ser<sup>173</sup>, in which a "charge-relay system" allows the His<sup>41</sup> and Asp<sup>88</sup> to transiently bind a proton from the Ser<sup>173</sup> which becomes a powerful nucleophile that attacks the peptide bond in the target protein. As its name suggests, NE is found in larger abundance in the blood neutrophil where it is stored at 0.5-3 pg/cell in cytoplasmic azurophilic granules, and rapidly released when the neutrophil is activated.

NE is classified as an "elastase" (EC 3.4.21.11) because it is one of a small group of proteases capable of attacking insoluble elastin, a highly cross-linked rubber-like macromolecule that modulates the structural integrity and mechanical properties of connective tissue matrixes. However, despite the specificity of its name, NE can also destroy most other matrix components (including collagen types I-IV, fibronectin and proteoglycans), as well as coagulation factors, immunoglobulins, complement components, and *E. coli* cell walls. As such, it has been hypothesized that NE plays a role in normal tissue turnover, the clearing of extracellular macromolecular debris during wound healing, the regulation of coagulation and immune responses, and antibacterial defenses. In the context of its powerful destructive capabilities, NE is considered to be the major proteolytic enzyme causing the progressive destruction of the alveolar walls that results in the emphysema associated with  $\alpha$ 1-antitrypsin deficiency and with cigarette smoking.

Although NE is carried and released by neutrophils, it is not clear that neutrophils can synthesize this enzyme. Furthermore, despite the specificity of the name "neutrophil" elastase, there is evidence that bone marrow-derived cells other than those of the myelocytic lineage are capable of expressing the NE gene. For example, NE has been recovered from the cells of the mononuclear phagocyte lineage, including blood monocytes and alveolar macrophages as well as U937, a cell line with many characteristics of the mononuclear phagocyte-lineage. From this evidence, and with the knowledge that neutrophils and mononuclear phagocytes are derived from a common bone marrow precursor, it is likely that the NE gene is expressed in the precursor marrow cells committed to the myelomonocytic lineage of cells, and that the cells of this lineage that are released into the blood have lost, or are in the process of losing, this capability.

To help to define what cells are capable of expressing the NE gene, we have isolated a human NE cDNA from cells known to produce NE and, using this cDNA as a probe, evaluated a variety of cells in which functional NE has been observed for presence of NE mRNA transcripts. A 0.65 kb cDNA (pPB15) complementary to the coding region of the NE gene was cloned from the cell line U937 using an oligonucleotide based on the known NE protein sequence. The sequence of pPB15 demonstrated that it coded for the 173 C-terminal residues of the 218 amino acids that comprise the mature NE protein, plus an additional 3' 60 bp prior to the in frame stop codon, suggesting the NE mRNA



contains sequences for a 20 residue C-terminal "pro" peptide that is not found in the mature protein. Northern analysis using <sup>32</sup>P-labeled pPB15 as a probe revealed that neutrophils do not contain detectable NE mRNA transcripts despite the fact that this cell carries large amounts of this protein. Furthermore, resting and activated blood monocytes also contained no detectable NE mRNA transcripts, although these cells also carry detectable NE. In contrast, bone marrow precursor cells contained NE transcripts, suggesting the NE gene is expressed in blood precursor cells. In this regard, evaluation of HL-60 cells, a human cell line with myelomonocytic lineage features, demonstrated NE transcripts in resting cells and increased NE mRNA levels when the cells were induced toward the myelocytic lineage with DMSO. However, when the HL-60 cells were induced toward the monocytic lineage with PMA, NE transcripts were lost even though transcripts for interleukin-1 $\beta$  were plentiful. Together, these observations are consistent with the concept that the NE gene is not expressed in the blood cells that carry the protein, but in bone marrow precursors that express NE transcripts about the time of commitment to the myelocytic series.

The gene for human neutrophil elastase (NE) was cloned from a genomic DNA library of a normal individual. The NE gene consists of 5 exons and 4 introns included in a single copy 4 kb segment of chromosome 11 at q14. The coding exons of the NE gene predict a primary translation product of 267 residues including a 29 residue N-terminal precursor peptide and a 20 residue C-terminal precursor peptide. Analysis of the N-terminal peptide sequence suggests it contains a 27 residue "pre" signal peptide followed by a "pro<sub>N</sub>" dipeptide, similar to that of other blood cell lysosomal proteases. The sequences for the mature 218 residue NE protein are included in exon II-V. The 5' flanking region of the gene includes typical TATA, CAAT, and GC sequences within 61 bp of the cap site. The sequence 1.5 kb 5' to exon I contains several interesting repetitive sequences including six tandem repeats of unique 52 or 53 bp sequences. The 5' flanking region also contains a 19 bp segment with 90% homology to a segment of the 5' flanking region of the human myeloperoxidase (MPO) gene, a gene also expressed in bone marrow precursor cells and a protein stored in the same neutrophil granules as NE. In addition, like the MPO gene, the NE 5' flanking region has several regions with  $\geq 75\%$  homology to sequences 5' to c-myc, but there is no overlap between the NE-c-myc and MPO-c-myc homologous sequences.

$\alpha$ 1AT deficiency is a genetic disorder characterized by low serum levels of  $\alpha$ 1AT and a high risk for the development of emphysema.  $\alpha$ 1AT is the principal inhibitor of neutrophil elastase, such that a deficiency of  $\alpha$ 1AT results in insufficient anti-elastase protection in the lower respiratory tract, thus allowing neutrophil elastase to destroy alveolar structures.  $\alpha$ 1AT is a 52 kDa 394 amino acid, single chain glycoprotein normally present in serum at 150-350 mg/dl. The  $\alpha$ 1AT gene, composed of 7 exons dispersed over 12 kb of chromosomal segment 14q31-32.3, is expressed in hepatocytes and mononuclear phagocytes. The  $\alpha$ 1AT protein, a member of the class of protease inhibitors proteins known as Serpins (Serine Protease Inhibitors), is a globular molecule composed of 9 alpha-helices and 3 beta-pleated sheets. The major function of  $\alpha$ 1AT is to inhibit neutrophil elastase;  $\alpha$ 1AT does so through an active site centered around Met<sup>358</sup> contained within an external stressed loop on the surface of the molecule.  $\alpha$ 1AT is a highly pleomorphic protein with greater than 75 variants determined at the protein and/or gene level. These



variants can be categorized into four groups according to their serum  $\alpha$ AT level and function; normal, deficient, dysfunctional, and absent. There are two important salt bridges within the  $\alpha$ AT molecule (Glu<sup>342</sup>--Lys<sup>290</sup>; Glu<sup>263</sup>--Lys<sup>387</sup>); mutations in the  $\alpha$ AT gene causing disruption of either salt bridge cause distinct molecular pathology resulting in reduced serum  $\alpha$ AT levels. Clinically relevant variants can be distinguished by a combination of isoelectric focusing of serum, restriction fragment length analysis of genomic DNA, oligonucleotide probes, and direct sequencing of the variant  $\alpha$ AT genes.

The normal M2 variant of  $\alpha$ AT was cloned from a genomic DNA library of an individual homozygous for this allele. Sequencing of all coding exons of the M2 gene revealed it was identical to the common M1(Val<sup>213</sup>) gene except for two bases [M1(Val<sup>213</sup>) CGT Arg<sup>101</sup>, M2 CAT His<sup>101</sup>; M1(Val<sup>213</sup>) GAA Glu<sup>376</sup>, M2 GAC Asp<sup>376</sup>]. Analysis of the sequence of the M1(Val<sup>213</sup>) and M2 genes around residue 101 revealed the M1 Arg<sup>101</sup>-->M2 His<sup>101</sup> caused a loss of the cutting site for the restriction endonuclease RsaI. Using this enzyme, as well as 19-mer oligonucleotide probes centered at residues 101 and 376, evaluation of genomic DNA from 22 M1 alleles and 14 M2 alleles revealed that residue 101 was Arg in all M1 alleles and His in all M2 alleles, while residue 376 was Glu in all M1 alleles and Asp in all M2 alleles. Despite the differences in sequence at two amino acids, the M1(Val<sup>213</sup>) and M2 proteins function similarly as assessed by quantification of the association rate constant of each for their natural substrate neutrophil elastase. In the context that there are two mutations separating the M1(Val<sup>213</sup>) and M2 alleles, it is likely that there is another  $\alpha$ AT variant that was an intermediate in the evolution of these genes.

An  $\alpha$ AT gene is defined as "Null" when no  $\alpha$ AT in serum is attributed to that  $\alpha$ AT gene. Although all  $\alpha$ AT Null genes have identical phenotypic consequences (i.e. no detectable  $\alpha$ AT in the serum), different genotypic mechanisms can cause the Null state. We have studied the molecular basis for the  $\alpha$ AT gene Null<sub>mattawa</sub>, identified and cloned from genomic DNA of an individual with the Null-Null phenotype and emphysema resulting from the heterozygous inheritance of the Null<sub>mattawa</sub> and Null<sub>bellingham</sub> genes. Sequencing of exons I-V and all exon-intron junctions of the Null<sub>mattawa</sub> gene demonstrated it was identical to the common normal M1(Val<sup>213</sup>)  $\alpha$ AT gene except for the insertion of a single nucleotide within the coding region of exon V causing a 3' frameshift with generation of a premature stop signal. Family analysis using oligonucleotide probes specific for the Null<sub>mattawa</sub> sequence demonstrated the gene was inherited in an autosomal fashion. Examination of blood monocytes demonstrated that a normal sized 1.8 kb  $\alpha$ AT mRNA transcript is associated with the Null<sub>mattawa</sub> gene and in vitro translation of mRNA with the Null<sub>mattawa</sub> mutation showed it translated at a normal rate but produced a truncated  $\alpha$ AT protein. In contrast, [<sup>35</sup>S]methionine labeling of the Null<sub>mattawa</sub> monocytes demonstrated no detectable intracellular or secreted  $\alpha$ AT. Additionally, retroviral transfer of the  $\alpha$ AT Null<sub>mattawa</sub> cDNA to murine fibroblasts demonstrated that the abnormality in  $\alpha$ AT biosynthesis could be reproduced in a cell that does not normally synthesize human  $\alpha$ AT. These findings are consistent with the concept that the molecular pathophysiology of Null<sub>mattawa</sub> is likely manifested at a post-translational level. The identification of the Null<sub>mattawa</sub> gene supports the concept that "Null"  $\alpha$ AT alleles represent a heterogenous group in which very different mecha-





nisms cause the identical phenotypic state.

The S-type (GAA Glu<sup>264</sup>->GTA Val<sup>264</sup>)  $\alpha$ 1AT deficiency allele results in  $\alpha$ 1AT levels 50% normal. To evaluate the hypothesis that the S-type deficiency results from intracellular instability of the S-type  $\alpha$ 1AT protein consequent to the A->T  $\alpha$ 1AT gene substitution, murine fibroblasts were modified to synthesize and secrete human  $\alpha$ 1AT by permanent integration of S-type or normal M1-type  $\alpha$ 1AT cDNA utilizing the N2 retroviral vector. Pulse-chase labeling studies with <sup>35</sup>S-methionine demonstrated that polyclonal populations of each cell type secreted a normal sized 52 kDa human  $\alpha$ 1AT. However, S-type cells had reduced secretion of  $\alpha$ 1AT compared to M1-type cells (S 45 $\pm$ 5% of M1, p<0.01). Since oligosaccharide side chains contribute to the stability of glycoproteins, inhibition of oligosaccharide side chain addition was utilized to exaggerate possible abnormalities in biosynthesis of the S-type protein. Treatment with tunicamycin (10  $\mu$ g/ml), an inhibitor of core oligosaccharide addition, resulted in secretion of a 46 kDa nonglycosylated  $\alpha$ 1AT by both cell populations, but the relative reduction in secretion by treated cells compared to untreated controls was markedly greater for S-type cells than for M1-type cells (M1+tunicamycin 87 $\pm$ 20% vs untreated, S+tunicamycin 2 $\pm$ 2% vs untreated; p<0.01). Thus, the S mutation is associated with diminished  $\alpha$ 1AT secretion, an effect that is markedly exaggerated if the molecule is not glycosylated. These observations suggest that the stabilizing effect of oligosaccharides is more critical to S-type than M1-type  $\alpha$ 1AT, and thus the S protein is less stable within the cell due to the Glu<sup>264</sup>->Val<sup>264</sup> substitution.

M<sub>procida</sub>, a rare  $\alpha$ 1AT allele associated with  $\alpha$ 1AT serum levels less than 10 mg/dl (normal 150-350 mg/dl), codes for an  $\alpha$ 1AT molecule that focuses on immobilized pH gradient isoelectric gels slightly cathodal to the common normal M1(Val<sup>213</sup>) protein. On a per molecule basis, M<sub>procida</sub> has a mildly reduced function as an inhibitor, with an association rate constant for human neutrophil elastase of 7.0 $\pm$ 0.1 x 10<sup>6</sup> M<sup>-1</sup>sec<sup>-1</sup> [normal M1(Val<sup>213</sup>) 9.3 $\pm$ 0.8 x 10<sup>6</sup>, p<0.01]. The M<sub>procida</sub> molecule behaves normally in vivo with a half-life similar to normal M1  $\alpha$ 1AT molecules. Restriction endonuclease mapping demonstrated that the cloned M<sub>procida</sub> gene was grossly intact. Sequencing of all the exons, exon-intron junctions, and the major promoter region demonstrated M<sub>procida</sub> to be identical to the M1(Val<sup>213</sup>) gene except for a single base substitution in exon II coding for amino acid 41 of the mature protein [M1(Val<sup>213</sup>) Leu<sup>41</sup> CTG->M<sub>procida</sub> Pro<sup>41</sup> CCG]. Usefully, the coding sequence of the  $\alpha$ 1AT residues 40-41 is recognized by the restriction endonuclease PvuII so that using a probe corresponding to this region of exon II, the M<sub>procida</sub> mutation can be rapidly identified by Southern analysis. Evaluation of the crystallographic structure of  $\alpha$ 1AT suggests the Leu<sup>41</sup> to Pro<sup>41</sup> mutation may disrupt  $\alpha$ -helix A in the region of Pro<sup>21</sup>-Ser<sup>45</sup>, suggesting the possibility that the  $\alpha$ 1AT M<sub>procida</sub> molecule is unstable and degraded intracellularly prior to secretion.

Homozygous inheritance of the Z-type  $\alpha$ 1AT gene (GAG Glu<sup>342</sup>->AAG Lys<sup>342</sup>) results in the most common form of  $\alpha$ 1AT deficiency. It is known that  $\alpha$ 1AT synthesizing cells of  $\alpha$ 1AT deficient individuals have normal  $\alpha$ 1AT mRNA levels but that  $\alpha$ 1AT secretion is markedly reduced secondary to accumulation of newly synthesized  $\alpha$ 1AT in the rough endoplasmic reticulum. Crystallographic analysis of  $\alpha$ 1AT predicts that in normal  $\alpha$ 1AT, a negatively charged Glu<sup>342</sup> is



adjacent to positively charged Lys<sup>290</sup>, and thus the Glu<sup>342</sup>-->Lys<sup>342</sup> Z mutation results in the loss of a normal 342---290 salt bridge, an event that may contribute to the intracellular aggregation of the Z molecule. In this context, we hypothesized that by creating a second mutation in the  $\alpha$ 1AT gene to change the positively charged Lys<sup>290</sup> to a negatively charged Glu<sup>290</sup>, the secretion defect could be corrected. Utilizing an *in vitro* eukaryotic expression system to evaluate  $\alpha$ 1AT secretion directed by  $\alpha$ 1AT cDNAs modified by site directed mutagenesis, the profound defect in  $\alpha$ 1AT secretion associated with the Z gene was reproduced. Strikingly, when a second mutation (AAG Lys<sup>290</sup>-->GAG Glu<sup>290</sup>) was added to the Z type cDNA, the resulting gene directed the synthesis and secretion of amounts of  $\alpha$ 1AT similar to that directed by the normal  $\alpha$ 1AT cDNA i.e., the biologic abnormalities characterizing this human hereditary disease can be corrected by inserting an additional mutation in a distant codon in the same gene.

Cosmid clones containing  $\alpha$ 1AT gene sequences were observed to contain  $\alpha$ 1AT-like sequences approximately 12 kb downstream of the authentic  $\alpha$ 1AT gene. Restriction mapping suggested the  $\alpha$ 1AT-like gene lacks promoter sequences. Cosmid clones from one library contained a truncated  $\alpha$ 1AT-like gene with a deletion encompassing 1745 bp, including the whole exon IV and part of exon V. Sequencing of exon II of this truncated gene revealed a nucleotide homology of 76% but included critical mutations in the start codon (ATG -> ATA) and the 3' exon-intron junction. These results strongly suggest that the truncated  $\alpha$ 1AT-like gene is a pseudogene.

Specific treatment of  $\alpha$ 1AT is available in the form of weekly intravenous infusions of human plasma  $\alpha$ 1AT, which effectively reconstitute the anti-elastase screen of the lung in these individuals. In an attempt to reduce the frequency of therapy, we have evaluated the ability of monthly infusions of  $\alpha$ 1AT to provide equivalent lower respiratory tract protection against neutrophil elastase. Intravenous infusion of 250 mg/kg  $\alpha$ 1AT at 28 day intervals to nine individuals with  $\alpha$ 1AT deficiency and emphysema was carried out for a period of 12 months. Mean serum  $\alpha$ 1AT levels rose from a pre-therapy level of 35 $\pm$ 10 mg/dl to a peak of 1312 $\pm$ 131 mg/dl 30 min post-infusion. Steady state serum  $\alpha$ 1AT levels post infusion progressively declined to a 28 day post-infusion level of 67 $\pm$ 10 mg/dl, a level 2-fold greater than the preinfusion level, and close to the theoretical threshold level for protection of 80 mg/dl. Importantly, serum  $\alpha$ 1AT levels exceeded the protective threshold for an average period of 25 days after each dose of  $\alpha$ 1AT. Furthermore, the post-infusion nadir lung epithelial lining fluid (ELF)  $\alpha$ 1AT level was 5-fold greater than the preinfusion level and the nadir ELF anti-neutrophil elastase capacity was also significantly elevated, nearly 3-fold above the preinfusion level. Thus during the entire dosing interval the lung is provided with adequate anti-neutrophil elastase protection. No adverse effects of monthly therapy were noted. These results indicate that monthly administration of human  $\alpha$ 1AT is fully capable of adequately augmenting serum and lung  $\alpha$ 1AT levels and anti-elastase capacity, and is therefore a rational alternative to weekly therapy.

The enormous surface area and slow permeability to macromolecules of the pulmonary epithelial surface suggest the lower respiratory tract may be a route by which  $\alpha$ 1AT could be directly targeted to the site where it is needed in  $\alpha$ 1AT deficiency. To examine this concept we evaluated the ability of an



aerosol of human  $\alpha$ 1AT to augment lung anti-elastase defenses in patients (n=7) with  $\alpha$ 1AT deficiency emphysema. The generator used created  $\alpha$ 1AT droplets sufficiently small ( $\leq 2 \mu\text{M}$  diameter) to reach the lower respiratory tract (LRT). Lung scintigraphic scanning following aerosol administration of mixtures of  $\alpha$ 1AT and technetium 99m-DTPA demonstrated that the aerosol reached the LRT and diffusely penetrated all lung zones. The ability of aerosol administration of  $\alpha$ 1AT to elevate lung epithelial lining fluid (ELF) anti-elastase defenses was evaluated using bronchoalveolar lavage with separate analyses of the first 2 of 5 aliquots (airway sample) and the final 3 aliquots (alveolar sample). One day after the aerosolization of 100 mg  $\alpha$ 1AT, lung ELF  $\alpha$ 1AT levels increased 6-fold in the alveolar samples ( $p < 0.01$ ). Aerosolization of 100 mg  $\alpha$ 1AT at 12 hr intervals for 3 days caused an 8-fold increase in alveolar  $\alpha$ 1AT levels (before  $0.29 \pm 0.07 \mu\text{M}$ , after  $2.54 \pm 0.81 \mu\text{M}$ ,  $p < 0.01$ ), and a 3-fold increase in alveolar anti-neutrophil elastase capacity (before  $0.71 \pm 0.11 \mu\text{M}$ , after  $2.34 \pm 0.82 \mu\text{M}$ ,  $p < 0.05$ ). Thus, the aerosol administration of  $\alpha$ 1AT results in significant augmentation of lung anti-elastase defenses suggesting that this is a feasible and rational approach to therapy in  $\alpha$ 1AT deficiency.

Gene therapy represents a future approach to preventing the emphysema associated with  $\alpha$ 1AT deficiency. Prior studies from our laboratory (Science 237:762, 1987) demonstrated that the ecotropic retroviral vector N2-FAT stably integrates the human  $\alpha$ 1AT cDNA under control of the constitutive early SV40 promoter into the genome of mouse fibroblasts, resulting in the production and secretion of glycosylated, functional human  $\alpha$ 1AT. To evaluate the feasibility of using retroviral technology to integrate the human  $\alpha$ 1AT cDNA into the human genome, the cell line PA317 was used to package N2-FAT into an amphotropic retrovirus capable of infecting human cells. The resulting high titer ( $2.5 \times 10^6/\text{ml}$ ) retrovirus was used to infect HeLa cells, a human cervical epithelial carcinoma cell line that does not normally express the  $\alpha$ 1AT gene. Capitalizing on the presence of the neomycin resistance gene in the N2-FAT construct, clones of HeLa cells infected with the PA317/FD3 virus (HeLa/ $\alpha$ 1) were selected by incubating the infected cells in medium containing the neomycin analog G418. Supernatants from HeLa/ $\alpha$ 1 clones were assayed for human  $\alpha$ 1AT by a human specific ELISA, and compared to those from uninfected HeLa cells, and 2 human hepatoma cell lines known to secrete  $\alpha$ 1AT, HEP3B2 and HEPG2. While the uninfected HeLa cells did not produce any detectable human  $\alpha$ 1AT, the HeLa/ $\alpha$ 1 clones produced  $110 \pm 10 \text{ ng}/10^6 \text{ cells per } 24 \text{ hrs}$ . In the same conditions, the HEP3B2 and the HEPG2 cells produced  $11,633 \pm 1695 \text{ ng}/10^6 \text{ cells}$  and  $8,622 \pm 225 \text{ ng}/10^6 \text{ cells}$  respectively. Thus, retroviral vectors can be used to introduce a normal human  $\alpha$ 1AT gene into the human genome, and, by use of an appropriate promoter, the  $\alpha$ 1AT gene can be expressed in cells that are not normally capable of expressing  $\alpha$ 1AT. Further refinements of this technology, including the use of other cell lines and the use of different promoters, as well as creating infected cells containing more than one copy of the  $\alpha$ 1AT gene may increase the protein production to the levels observed in the two hepatoma lines.

## II. Disorders characterized by extensive fibrosis of the lung parenchyma

These disorders represent a large subgroup of the interstitial lung disorders, chronic disorders in which derangements of the alveolar wall are accompanied by "fibrosis," a process in which the normal parenchyma is replaced by



mesenchymal cells and the collagenous matrix produced by these cells. In general, the inflammation of these disorders is dominated by alveolar macrophages, and to a lesser extent, neutrophils and/or eosinophils. Examples of these fibrotic disorders include idiopathic pulmonary fibrosis and asbestosis. The current concepts of the mechanisms of pulmonary fibrosis hold that the accumulation of fibroblasts and fibroblast products result from two general processes. First, there must be damage to the alveolar wall; in most cases this is mediated by products of inflammatory cells, including macrophages, neutrophils and eosinophils. Second, there is enhanced proliferation of mesenchymal cells in the alveolar walls driven by growth signals released in the local milieu.

Tissue macrophages, members of the mononuclear phagocyte system of bone marrow-derived cells present in most organs, are thought to play a central role in normal wound healing and pathologic tissue fibrosis by virtue of their ability to release a variety of polypeptide mediators that serve as growth factors for mesenchymal cells. In humans, most information regarding tissue macrophage production of mesenchymal growth factors relates to the alveolar macrophage, a tissue macrophage easily accessible by bronchoalveolar lavage. Human alveolar macrophages are known to be capable of expressing the genes for several defined growth factors including the *c-sis* gene [the B-chain of platelet-derived growth factor (PDGF)], fibronectin, insulin-like growth factor-1 (IGF-I), interleukin 1- $\beta$  and tumor necrosis factor. Furthermore, in the fibrotic lung diseases, alveolar macrophages are spontaneously releasing exaggerated amounts of PDGF, fibronectin, and IGF-I leading to the concept that the alveolar macrophage plays an important role in directing the exaggerated fibroblast accumulation in the alveolar walls that characterizes these disorders.

In the dual control model of the timing of growth factor action during the cell cycle of fibroblast proliferation, the concept has developed that fibroblasts require two signals to trigger growth, a "competence" signal acting early in G<sub>1</sub>, and a later "progression" signal acting late in G<sub>1</sub> to stimulate the cell to synthesize DNA and proliferate. In regard to growth factors released by alveolar macrophages, PDGF and fibronectin are capable of serving as "competence" factors for mesenchymal cells. These cells are also capable of producing a "progression" factor. In 1982, we described a polypeptide with "progression" activity released from activated alveolar macrophages. This mediator, termed "alveolar macrophage-derived growth factor" (or "AMDGF") had no "competence" activity, but in serum-free conditions was capable of stimulating PDGF or fibronectin-primed fibroblasts to move through G<sub>1</sub> and synthesize DNA within 8 hrs. Furthermore, in fibrotic lung disorders such as IPF and asbestosis, AMDGF was spontaneously released, but was not detectable in supernatants of normal resting alveolar macrophages. At the time of its discovery, evaluation of AMDGF suggested it was different from all other known growth factors. Specifically, in regards to the "progression" class of fibroblast growth factors, its apparent molecular mass insured it was not insulin, insulin-like growth factor-I (IGF-I, "somatomedin C") or insulin-like growth factor-II (IGF-II).

Attempts to further characterize AMDGF were hampered by the difficulty in obtaining sufficient numbers of alveolar macrophages necessary to purify the protein. However, several recent observations have led us to reevaluate the





possibility that AMDGF may be a member of the IGF-I family. First, "tissue-type" forms of IGF-I have been recently described, including a fibroblast type of IGF-I with a molecular mass of 21.5 kDa, and a Sertoli cell-produced type of IGF-I with a molecular mass of 25 kDa. Both of these tissue IGF-I-like molecules have molecular masses approximately 3-fold greater than the plasma form of IGF-I and in the same range as AMDGF. Second, studies in liver have shown that the IGF-I gene can be expressed in a variety of ways, utilizing alternative splicing to produce different IGF-I mRNA transcripts. Finally, in situ hybridization studies have demonstrated that most fetal tissues express the IGF-I gene, including the interstitium of the lung, one site where alveolar macrophages reside.

In this context, we have directed efforts toward evaluating the hypothesis that alveolar macrophages produce a progression-type growth factor for fibroblasts with properties that place it as a member of the IGF-I family. The data demonstrate that this is the case, and suggest that the mediator previously described as "alveolar macrophage-derived growth factor", is an IGF-I-type molecule. Partial purification of medium conditioned by activated alveolar macrophages using ion exchange and gel filtration chromatography revealed an IGF-I molecule as detected by an anti-IGF-I polyclonal antibody and that the specific activity of the progression-type growth activity tracked with the amount of IGF-I present. In a serum-free complementation test, the increase in fibroblast proliferation by alveolar macrophage IGF-I was significantly reduced in a dose-response manner with an anti-IGF-I monoclonal antibody. The alveolar macrophage IGF-I displaced  $^{125}\text{I}$ -IGF-I from its receptor in a binding assay utilizing human lung fibroblasts and it stimulated type I IGF receptors purified from human lung fibroblasts to phosphorylate a tyrosine-containing artificial substrate. In contrast to the 7.6 kDa serum type of IGF-I, gel chromatography revealed the alveolar macrophage-type IGF-I had an apparent molecular mass of 26 kDa, in the range of other tissue IGF-I's. Finally, fresh alveolar macrophages expressed IGF-I gene-related mRNA transcripts as detected by solution hybridization using a  $^{32}\text{P}$ -labeled riboprobe complementary to exons I-II-III of the IGF-I gene. Interestingly, normal, resting human alveolar macrophages, as well as macrophages recovered from the lower respiratory tract of individuals with chronic lung inflammation, constitutively express transcripts of the IGF-I gene. However, an IGF-I-type molecule is not spontaneously released by normal, resting alveolar macrophages. In contrast, alveolar macrophages recovered from the lungs of individuals with chronic inflammation in the lower respiratory tract spontaneously release significant amounts of IGF-I. Together, these observations suggest that tissue macrophages have a marked divergence of expression of the IGF-I gene at the mRNA and protein levels: while the gene apparently is constitutively expressed at the mRNA level, macrophages modulate expression of these transcripts such that an IGF-I-type protein is released only in inflammatory states, circumstances in which the IGF-I gene product likely plays a role in the localized proliferation of mesenchymal cells.

Transforming growth factor- $\beta$  (TGF- $\beta$ ) is a 25 kDa disulfide linked homodimer or heterodimer protein with a broad range of biologic functions. Originally described by its ability to reversibly transform the phenotype of NRK fibroblasts in culture in the presence of transforming growth factor- $\alpha$  or epidermal growth factor, it is now recognized that TGF- $\beta$  probably has a much wider biologic role in relation to the normal modulation of cell proliferation and



differentiation. In this regard, TGF- $\beta$  has been shown to inhibit the proliferation of T-lymphocytes and B-lymphocytes, epithelial cells, and vascular endothelial cells. TGF- $\beta$  also can inhibit diploid fibroblast and smooth muscle cell proliferation, although, in some experimental conditions, this versatile mediator can stimulate mesenchymal cell proliferation, suggesting the importance of the local milieu upon TGF- $\beta$  effects. Beside its action on cell division, TGF- $\beta$  stimulates the squamous differentiation of normal bronchial epithelial cells, augments fibroblast production of collagen and fibronectin, and is a chemoattractant for fibroblasts and blood monocytes. In recent studies of the armamentarium of genes available to the human alveolar macrophage in its role in releasing polypeptide mediators capable of modulating the growth of lung parenchymal cells, we observed that normal resting human alveolar macrophages constitutively express the TGF- $\beta$  gene and release TGF- $\beta$ . In this regard, and in the context of the knowledge that large numbers of alveolar macrophages reside on the epithelial surface of the lower respiratory tract, we hypothesized that the epithelial fluid lining this surface may normally contain this mediator. Strikingly, the data demonstrate that not only is TGF- $\beta$  present in human lower respiratory tract epithelial lining fluid (ELF), but that the amounts of TGF- $\beta$  in ELF are sufficiently high such that, if active, they should have significant biologic effects in the local milieu. While plasma contained small amounts of TGF- $\beta$ , the concentrations of TGF- $\beta$  in normal ELF were in the 200 to 300 pM range, more than 15-fold higher. This ELF TGF- $\beta$  had similar physical characteristics to purified human platelet TGF- $\beta$ , competed with platelet TGF- $\beta$  for its receptor on A549 carcinoma cells and stimulated the anchorage-independent growth of NRK cells in soft agar in the presence of epidermal growth factor. Furthermore, ELF TGF- $\beta$  suppressed diploid lung fibroblast proliferation in a dose-dependent fashion similar to platelet TGF- $\beta$ . In the context of these observations and with the known biologic properties of this molecule, TGF- $\beta$  in ELF has the potential to play a role in a variety of cellular processes in the lower respiratory tract.

Tumor necrosis factor (TNF; also referred to as "cachectin") is a multimeric protein comprised of two or more identical 17 kDa disulfide subunits. Originally described because of its cytotoxicity for certain tumor cell lines, TNF is now recognized to have a broad range of activities associated with acute and chronic inflammatory states and has been implicated as the exogenous mediator of endotoxic shock. In addition, TNF is thought to be part of the nonspecific antineoplastic and antiviral systems, and it is capable of acting as a growth stimulus for mesenchymal cells. It has been recognized for some time that activated human blood monocytes are capable of synthesizing and releasing TNF and the TNF cDNA was cloned from the HL-60 human myelomonocytic cell line. Together with the observation that various animal macrophage cell lines also produce TNF, the knowledge that monocytes activated by endotoxin release TNF has led to the concepts that mononuclear phagocytes are a major source of TNF and that TNF represents a major mechanism by which macrophages attack tumor cells. In the context of these observations, it is reasonable to hypothesize that human alveolar macrophages, the pulmonary representatives of the mononuclear phagocyte system, are capable of expressing the TNF gene and synthesizing and releasing TNF. In this regard, it is recognized that, in the lung, alveolar macrophages accumulate at sites of infection and inflammation, play a role in local defense against tumors, and are capable of releasing mediators that mediate local tissue fibrosis, all of which are activities



that have been attributed, at least in part, to TNF. To evaluate the relative capacity of human tissue macrophages to produce this mediator, alveolar macrophages and blood monocytes from the same normal individuals were activated with lipopolysaccharide (LPS) and evaluated for TNF release and TNF mRNA transcript levels. Resting alveolar macrophages did not express TNF mRNA transcripts or release TNF. However, when activated, alveolar macrophages expressed TNF transcripts and synthesized and released TNF as evidenced by the presence of a 28 kDa mediator in LPS activated alveolar macrophage supernatants that had cytotoxic activity for L-929 cells that was abrogated by anti-TNF antibodies and that co-eluted with a pure TNF standard on a molecular sieve column. Interestingly, activated alveolar macrophages released several-fold more TNF than did autologous blood monocytes stimulated in a similar fashion and, in parallel, the alveolar macrophages expressed more TNF mRNA transcripts than activated blood monocytes. Thus, the ability to express the TNF gene and to release TNF apparently increases during maturation of blood monocytes into alveolar macrophages, suggesting that the release of TNF in the local milieu by activated tissue macrophages may be much more significant than the release of this mediator by circulating blood monocytes.

Iron is an essential element for growth and survival of eukaryotic cells. Under normal circumstances iron gains intracellular access by the carrier protein transferrin through its interaction with a specific 180 kd glycoprotein cell surface receptor. Once the iron-transferrin complex binds to the transferrin receptor, both are internalized, the iron is released within the cell, and the transferrin with its receptor cycles back to the cell surface, where the transferrin is released. In this regard, it is reasonable to conclude that, given ample supplies of iron-transferrin in the extracellular milieu, the number and function of transferrin receptors play a major role in modulating the movement of iron into the cell. Consistent with this concept, numerous studies have demonstrated that transferrin receptor expression is upregulated under circumstances associated with an increased intracellular demand of iron, including cell proliferation. These observations have led to the concept that the level of transferrin receptor expression may play a role in the control of cell proliferation. In this regard, it has been demonstrated that cell surface transferrin receptor expression is a prerequisite for DNA synthesis in mitogen-stimulated human T-lymphocytes. In these cells, binding of the T-cell growth factor interleukin-2 to its receptor causes the sequential induction of transferrin receptor expression followed by DNA synthesis, while blocking of the transferrin receptor prevents DNA synthesis. From these studies it has been concluded that upregulation of transferrin receptor expression occurs following growth factor interactions with their target cells, and that such upregulation is necessary for cell proliferation to occur. In the context of these findings, we have carried out studies to determine whether such concepts are applicable to cells other than lymphocytes. Specifically, we have asked whether the expression of the transferrin receptor gene is modulated when normal human diploid fibroblasts are signaled to proliferate. The data demonstrate that growth signals induce fibroblasts to increase transferrin receptor mRNA levels and transferrin receptor numbers, but unlike lymphocytes, blocking of the transferrin receptor does not prevent peptide growth factor induced proliferation of fibroblasts. Furthermore, the upregulation of transferrin receptor mRNA levels by growth factors can occur in the absence of proliferation and depletion of intracellular iron stores with the iron chelator desferoxamine results in the marked potentia-



tion of this growth factor effect. Thus, in contrast to what has been observed with mitogen-stimulated human lymphocytes, transferrin receptor expression and upregulation do not appear to be required for the transduction of growth factor signals which trigger HFL-1 fibroblast proliferation. Rather, growth factor-induced upregulation of transferrin receptor gene expression in fibroblasts likely represents, at least in part, an "enhancer system" for providing the cell with iron when needed.

Transmission electron microscopic studies were made to determine the morphological features, total numbers and relative proportions of the different types of alveolar mesenchymal cells, including fibroblasts, myofibroblasts, pericytes, smooth muscle cells, and undifferentiated and mixed mesenchymal cells, present in open lung biopsy specimens from nonsmoking normal individuals (n=5) and from nonsmoking patients (n=9) with idiopathic pulmonary fibrosis (IPF) in midcourse. There were profound quantitative and qualitative differences between the mesenchymal cell populations in normal and IPF lungs. In normals, mesenchymal cells represented  $29 \pm 2\%$  of all parenchymal cells, with myofibroblasts being the predominant cell type. Mesenchymal cells, especially myofibroblasts, had elongated cytoplasmic processes allowing for contacts with other mesenchymal cells, thus forming an interconnected cellular network. Mesenchymal cells also had focal contacts with epithelial and endothelial cells. In fibrotic lungs, mesenchymal cells represented  $52 \pm 2\%$  of all parenchymal cells, and the numbers of all types of mesenchymal cells were increased compared to those in normal lungs. Marked shifts in the relative proportions of cells were also found, including a ten-fold increase in smooth muscle cells, a marked increase in mixed cells, and a decrease in myofibroblasts. Mitotic figures in mesenchymal cells were seen only in cells with the morphological characteristics of fibroblasts. Morphologically, most cell types were altered. The most important qualitative changes in IPF affected the myofibroblasts and resulted in loss of alveolar stability and in derangements of cell-to-cell contacts.

### III. Disorders characterized by granulomata in the alveolar walls.

Disorders characterized by granulomata in the alveolar walls are a subgroup of the interstitial lung disorders. Although occasionally associated with progressive fibrosis of the lung parenchyma late in their course, these disorders are usually more benign and are universally characterized by large numbers of T-lymphocytes that dominate the inflammation and direct the formation of granulomata. Together, the T-cells and granulomata cause dysfunction by their presence which distorts the alveolar, bronchial, and vascular walls, thus modifying the intimate relationship between air and blood. Examples of these disorders include sarcoidosis and berylliosis.

Sarcoidosis is a non-malignant, chronic, systemic disorder characterized by a T-lymphocyte and mononuclear phagocyte inflammatory process associated with granuloma formation in affected organs. The etiology of sarcoidosis is unknown, but it is recognized that the T-cell plays a critical role in the pathogenesis of the disease. In this regard, evaluation of the T-lymphocytes in the lower respiratory tract of individuals with active sarcoidosis has shown that they are dominated by active helper/inducer T-lymphocytes that are releasing lymphokines, processes





fundamental to formation of immune granulomata. These T-cells accumulate in the lung, at least in part, because of processes that stimulate the activation and proliferation of helper/inducer T-lymphocytes in the local milieu. In the context of these observations, a central question in understanding the pathogenesis of sarcoidosis is to understand why T-lymphocytes accumulate at sites of disease such as the lower respiratory tract. Three general hypotheses can be proposed to explain the pathogenesis of these T-lymphocyte infiltrations in sarcoidosis. First, the accumulated T-cells may represent a polyclonal T-cell response, perhaps secondary to a generalized enhancement of T-helper cell processes or ineffective T-cell suppressor networks that are not antigen-specific. Second, the T-cells may accumulate secondary to a monoclonal or oligoclonal process such as that observed in malignancies in which a "transformed" cell with a growth advantage accumulates in tissues. Third, the T-cells may accumulate secondary to antigen-driven processes, in which the clonal expansion of antigen-specific T-cells together with the secondary expansion of populations of immunoregulatory and/or bystander T-cells results in a skewed distribution of T-cells. Since the mechanisms responsible for T-cell accumulation in these three categories are different, a characterization of the "clonality" of sarcoid T-cells is an important first step in understanding the pathogenesis of this disorder.

As an approach to this question, we have capitalized on the identification and cloning of the  $\beta$ -chain gene of the T-cell antigen receptor. Since the  $\beta$ -chain gene undergoes specific DNA rearrangement during normal T-cell ontogeny, these rearrangements serve as a genotypic marker for T-cells that can be detected by Southern blot analysis of DNA isolated from T-cells. Using this immunogenotypic approach, we evaluated T-cells from the lung and blood of patients with active sarcoidosis, inactive sarcoidosis and normals. The data clearly demonstrate that individuals with active pulmonary sarcoidosis accumulate enhanced numbers of clonally-diverse T-cells with uncommon partial ( $D_{\beta 1}J_{\beta 2}$ )  $\beta$ -gene rearrangements, suggesting there is a bias for T-cells with a restricted number of T-cell  $\beta$ -chain gene rearrangements in this chronic inflammatory state. In this regard, DNA from lung T-cells of 7 of 10 individuals with active sarcoidosis demonstrated  $\beta$ -chain gene rearrangements on Southern blot analysis consistent with partial  $D_{\beta 1}J_{\beta 2}$  rearrangements of the  $\beta$ -chain gene locus. In contrast, normal individuals and 5 of 5 cases of inactive pulmonary sarcoidosis had no detectable  $\beta$ -chain gene rearrangements among their lung T-cells. Interestingly, blood T-cell DNA of 6 of 10 individuals with active pulmonary sarcoidosis also demonstrated partial  $D_{\beta 1}J_{\beta 2}$  rearrangements of the  $\beta$ -chain gene, indicating a systemic nature of the T-cell processes associated with this disorder. Since the  $\beta$ -gene partial rearrangements of lung and blood T-cells of different individuals with sarcoidosis were different and not associated with unique, complete VDJ  $\beta$ -gene rearrangements, it is likely that a clonally-diverse population of T-cells with a highly restricted repertoire of  $\beta$ -chain gene partial rearrangements on one allele is involved in the chronic inflammatory processes that characterize this disorder.

With the knowledge that activated CD4+ T-cells play a central role in the pathogenesis of active sarcoidosis, we hypothesized that the T-cell



antigen receptor  $\beta$ -chain rearrangements would be found preferentially in the CD4+ subset of T-lymphocytes. To evaluate this hypothesis, the genotypic configuration of the T-cell antigen receptor  $\beta$ -chain was analyzed in DNA purified from CD4+ and CD8+ T-cells from patients with active pulmonary sarcoidosis. Interestingly, contrary to the initial hypothesis, DNA extracted from both CD4+ and CD8+ T-cell subpopulations, when digested with BamHI and evaluated with a  $\beta$ -chain constant region probe, demonstrated non-germline rearrangements similar to those in unfractionated T-lymphocytes of the same individuals. These results were confirmed using a probe of the  $J_{\beta 2}$ - $C_{\beta 2}$  intron of the T-cell antigen receptor  $\beta$ -gene. Importantly, when sarcoid T-cells were sorted using a monoclonal antibody WT31 (TCR-1), that detects the surface T-cell antigen receptor  $\alpha/\beta$  heterodimer, only T-cells expressing the T-cell antigen receptor  $\alpha/\beta$  heterodimer demonstrated non-germline T-cell antigen receptor  $\beta$ -gene rearrangements, i.e., the  $D_{\beta 1}J_{\beta 2}$  rearrangements occur only in sarcoid T-cells that have a productive T-cell antigen receptor  $\beta$ -chain gene rearrangement. Thus, non-germline T-cell antigen receptor  $\beta$ -gene rearrangements are found in both CD4+ and CD8+ sarcoid T-lymphocytes that express an  $\alpha/\beta$  T-cell antigen receptor heterodimer, suggesting that sarcoidosis is characterized by a population of functional CD4+ and CD8+ T-cells with a common, restricted pattern of T-cell antigen receptor  $\beta$ -gene rearrangements in their second, nonfunctional  $\beta$ -chain allele.

As an alternate approach to evaluating the biased T-cell rearrangements in sarcoidosis, we evaluated sarcoid lung and blood T-cell populations for usage of the  $V_{\beta 8}$  subfamily of  $D_{\beta}$  variable regions. We focused on  $V_{\beta 8}$  after a preliminary screen with a panel of T-cell clonotype-specific antibodies demonstrated a striking bias for T-cells expressing  $V_{\beta 8}$  surface determinants in a subgroup of individuals with this disease. An antibody (anti- $T_{i3A}$ , 5REX9H5) specific for the  $V_{\beta 8}$  subfamily was used to evaluate lung and blood T-cells in pulmonary sarcoidosis. Whereas normal individuals all had <5% of lung ( $n=7$ ) and/or blood ( $n=9$ ) lymphocytes that were  $T_{i3A}+$ , strikingly, a subgroup of individuals (8 of 21) with active pulmonary sarcoidosis had >7%  $T_{i3A}+$  lung and/or blood T-cells. Notably, among the subgroup, the proportions of  $T_{i3A}+$  lymphocytes in the lung were greater than the proportion of  $T_{i3A}+$  lymphocytes in blood, i.e., dual color cytometry demonstrated that both CD4+ and CD8+ T-cells expressed  $T_{i3A}+$  antigen receptors, although  $T_{i3A}+$  CD4+ lymphocytes were compartmentalized to lung while  $T_{i3A}+$  CD8+ lymphocytes were mostly partitioned to blood. Consistent with evidence of bias in the use of  $T_{i3A}+$   $\beta$ -chains at the cell surface, analysis with a  $^{32}P$ -labeled  $V_{\beta 8}$  gene probe ( $V_{\beta}REX$ ) demonstrated that lung T-lymphocytes of sarcoid patients contained higher amounts of  $V_{\beta 8}+$  mRNA levels than autologous blood T-cells. Interestingly, despite the bias in use of  $V_{\beta 8}$  gene elements in sarcoidosis, Southern analysis of sarcoid lung and blood T-cell DNA using the  $V_{\beta}REX$  gene probe demonstrated no evidence of clonal populations of T-cells with similar rearrangements or deletions of  $V_{\beta 8}$  gene elements. Together, these observations demonstrate a clear bias toward the use of at least one  $V_{\beta}$  region in a subgroup of individuals with sarcoidosis, suggesting that in this chronic, non-malignant disorder, T-cells likely accumulate secondary to external selective pressure, rather than in a random polyclonal clonal fashion or by clonal expansion of one or a few T-cell clones.



Among the various possible processes involved in the accumulation of the CD4+ T-cells in the lower respiratory tract, it is known that alveolar macrophages of individuals with sarcoidosis have an enhanced ability to present antigen compared to normal alveolar macrophages i.e., for a fixed amount of a specific antigen, sarcoid alveolar macrophages induce exaggerated proliferation of autologous or human leukocyte antigen (HLA) Class II matched heterologous T-cells. In this regard, independent of the specific antigens involved, enhanced antigen presentation may explain in part, why individuals with active sarcoidosis have exaggerated numbers of proliferating T-cells in the lower respiratory tract. Antigen presenting cells deliver two signals that play a central role in the immune response of CD4+ T-lymphocytes. First, antigen is processed and presented in conjunction with the appropriate HLA class II surface molecule. Second, the macrophage releases interleukin-1 (IL-1), a lymphokine that augments the activation and subsequent proliferation of lymphocytes. In the context of the knowledge that most evidence suggests IL-1 gene expression and/or release by sarcoid alveolar macrophages is not up-regulated, it is possible that the ability of sarcoid alveolar macrophages to present antigen in an exaggerated fashion could result from an increased expression of HLA class II antigens on the surface of alveolar macrophages of these individuals. Furthermore, the expression of class II molecules by mononuclear phagocytes is known to be up-regulated by interferon- $\gamma$ , an immunoregulatory molecule known to be released in an exaggerated fashion in the sarcoid lung. On the basis of these observations, it is reasonable to hypothesize that the expression of the class II surface antigens may be up-regulated by alveolar macrophages in individuals with active sarcoidosis. To evaluate this question, we examined both normal and sarcoid alveolar macrophages for mRNA transcripts of class II molecules and have quantified surface expression of class II molecules (HLA-DR, DQ, and DP) on these macrophages. Northern blot and dot blot analysis with  $^{32}\text{P}$ -labeled cDNA probes for HLA-DR, DQ, and DP genes revealed that both normal and sarcoid alveolar macrophages contain class II mRNA transcripts, but that the levels in sarcoid and normal alveolar macrophages are similar ( $p > 0.3$ , all comparisons). Furthermore, evaluation of surface expression of class II molecules with monoclonal antibodies and flow cytometry analysis demonstrated that alveolar macrophages of both sarcoidosis patients and normals express all three class II molecules, DR, DQ and DP, but there were no significant differences between sarcoid and normals in the proportions of macrophages expressing these surface molecules. However, there was a significantly higher surface density of class II molecules on sarcoid alveolar macrophages ( $p < 0.05$ , all comparisons sarcoid to normal). Thus, enhanced surface expression of class II molecules is observed on sarcoid alveolar macrophages, consistent with the concept that the expression of class II molecules may play an important role in the ability of sarcoid alveolar macrophages to present antigen and stimulate lymphocytes, explaining in part, the accumulation of activated helper T-cells in sarcoidosis.

Chronic exposure to beryllium (Be) is known to cause a granulomatous lung disorder, distinguishable from sarcoidosis by the presence of Be in the lung parenchyma and by the ability of lung T-cells to proliferate in



response to Be. However, animal studies suggest that Be can act as a mitogen and adjuvant, and thus it is not known whether the T-cell responsiveness to Be exhibited by these individuals is a non-specific response or an antigen-driven immune process. To prove that Be truly acts as an antigen in this disease, lung T-cells from berylliosis patients (n=5) were cultured in the presence of Be and interleukin-2 to determine if Be-specific human T-cell lines could be developed. After 3 wks, the T-cell lines were tested for responsiveness to BeSO<sub>4</sub> and to the common recall antigen tetanus toxoid (TT). While all lines responded to Be [stimulation index (SI) 48±20], they did not respond to TT (SI<1); in contrast, autologous TT-specific T-cell lines responded to TT (SI 19±13) but not to Be (SI<1). To evaluate the response to Be at the single cell level, T-cell clones were derived by limiting dilution from the Be-specific lung T-cell lines. These clones: (1) were monoclonal as determined by Southern analysis of the T-cell receptor  $\beta$ -chain rearrangement pattern using a <sup>32</sup>P-labeled constant region DNA probe; (2) displayed the helper T-cell phenotype (CD4+, CD8-); and (3) responded to BeSO<sub>4</sub> in a dose dependent manner. Evaluation of the Be-driven proliferation of these clones using an anti-class II major histocompatibility complex (MHC) antibody (9.49) or anti-class I MHC antibody (W6/32) showed that Be-stimulated proliferation was clearly class II MHC restricted (% inhibition: anti-class II 100±2%; anti-class I 7±30%, p<0.01) while PHA-stimulated proliferation was not (% inhibition: anti-class II <1%; anti-class I 9±2%) as expected for antigen specific helper/inducer T-cells. Together these observations strongly support the concept that in individuals with berylliosis, Be does act as an antigen, driving the proliferation of the helper/inducer T-cell subset in an antigen-specific fashion.

The CD45 Leukocyte Common Antigen gene transcript undergoes alternative splicing to generate a protein family, including the 205, 220 kDa antigens (2H4+) expressed on autologous MLR-responsive/suppressor-inducer T-cells and the 180 kDa antigen (UCHL1+) expressed on antigen-primed helper T-cells. To test if T-cells on the epithelial surface are dominated by antigen-primed T-cells, lung and blood T-cells from normals (n=5) were evaluated by two color FACS analysis with 2H4, UCHL1 and Leu4 (CD3). While blood CD3+ T-cells were 51±12% UCHL1+ and 46±15% 2H4+, lung T-cells were 92±2% UCHL1+ and only 16±11% 2H4+ (p<0.01 both compared to blood) i.e., normal lung T-cells appear "antigen-primed." In this context, we hypothesized that in berylliosis, a specific antigen (Be-protein complex)-driven disorder characterized by helper T-cell accumulation in the lung, the accumulated lung T-cells would be UCHL1+. Bronchoalveolar lavage of berylliosis patients (n=6) revealed 20±12-fold more lung T-cells than normal (p<0.01). Of these almost all were UCHL1+ (95±5%, 2H4 4±3%), while blood T-cells were 45±9% UCHL1+ and 55±6% 2H4+ i.e., Be-driven lung T-cell activation expands antigen-primed UCHL1+ and not 2H4+ T-cells. This response was antigen-specific because: 1) Be caused marked lung, but not blood T-cell proliferation in vitro (lung stimulation index 82±23, blood 5±3, p<0.01); 2) lung T-cell derived Be-specific T-cell lines were 100% UCHL1+/2H4- and responded to Be in a specific HLA-class II restricted manner (anti-class II Ab 9.49, 100±2% inhibition; anti-class I Ab W6/32, 7±30%, p<0.01). These data suggest that T-cells on epithelial surfaces are dominated by antigen-primed





T-cells and that such cells accumulate accompanying chronic exposure to specific antigens.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02407-14 PB

PERIOD COVERED

October 1, 1987 - September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Destructive Lung Disease

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: R.G. Crystal Chief Pulmonary Branch, NHLBI

Others: T. Abe Visiting Fellow Pulmonary Branch, NHLBI  
M. Brantly Senior Staff Fellow Pulmonary Branch, NHLBI  
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R.C. Hubbard Senior Staff Fellow Pulmonary Branch, NHLBI

COOPERATING UNITS (if any)

Michael Courtney - Transgene, Strasbourg, France  
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LAB/BRANCH

Pulmonary Branch

SECTION

INSTITUTE AND LOCATION NHLBI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS: 12.7	PROFESSIONAL: 12.5	OTHER: 0.2
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CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

There are 2 million individuals in the U.S.A. with emphysema. Two per-  
cent develop the disease because of inheritance of a deficiency of alpha  
1-antitrypsin ( $\alpha$ 1AT), an antiprotease that protects the lower respira-  
tory tract from destruction mediated by elastase released by neutroph-  
ils. The neutrophil elastase cDNA and genomic DNA have been cloned and  
sequenced and the site expression of this gene has been localized to  
promyelocytes in bone marrow. Cloning, sequencing and oligonucleotides  
have been used to detect specific mutations in the  $\alpha$ 1AT gene causing the  
deficiency states. The "null"  $\alpha$ 1AT state is associated with an intact  
gene, but contains various different mutations causing stop codons in  
the  $\alpha$ 1AT mRNA. Therapy of  $\alpha$ 1AT deficiency with  $\alpha$ 1AT purified from pooled  
plasma has demonstrated that the anti-neutrophil-elastase defenses of  
the lung can be re-established with intermittent intravenous administra-  
tion of  $\alpha$ 1AT weekly or monthly as well as with intermittent aerosol  
administration daily.

768



## NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02533-04 PB

## PERIOD COVERED

October 1, 1987 - September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Fibrotic Disorders

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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Others: Z. Borok Staff Fellow Pulmonary Branch, NHLBI  
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## COOPERATING UNITS (if any)

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## LAB/BRANCH

Pulmonary Branch

## SECTION

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland

## TOTAL MAN-YEARS:

11

## PROFESSIONAL:

10

## OTHER:

1

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The fibrotic lung disorders represent 15% of the non-infectious, non-malignant lung diseases; they are often progressive and can be fatal. The fibrosis results from damage caused by inflammatory cells and subsequent proliferation of mesenchymal cells, driven by mediators released by alveolar macrophages. The primary mediators are platelet-derived growth factor, fibronectin and insulin-like growth factor-I. Other mediators released by stimulated alveolar macrophages include tumor necrosis factor, transforming growth factor- $\beta$  and interleukin 1. With knowledge of the specific processes involved, strategies can be developed to modulate these mediators as therapy for these disorders.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02534-04 PB

## PERIOD COVERED

October 1, 1987 - September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Granulomatous Disorders

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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## COOPERATING UNITS (if any)

V.J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, IR,  
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## LAB/BRANCH

Pulmonary Branch

## SECTION

INSTITUTE AND LOCATION NHLBI, NIH, Bethesda, Maryland

## TOTAL MAN-YEARS:

10.5

## PROFESSIONAL:

10.0

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The granulomatous lung disorders occur in 20 to 50 per 100,000 of the U.S.A. population. The "model" disorder of this group is sarcoidosis, a disease characterized by the accumulation of activated helper T-lymphocytes at sites of disease. These T-cells spontaneously express the interleukin-2 gene, thus driving T-cells in the local milieu to proliferate. Evaluation of T-cells in these individuals demonstrates clonal populations, some with partial DJ rearrangements of the  $\beta$ -chain of the T-cell antigen receptor and others using specific variable regions of the  $\beta$ -chain. Evaluation of lung T-cells of individuals with chronic beryllium exposure demonstrated that beryllium clearly acts as an antigen, driving proliferation of helper T-cells in this disorder.





ANNUAL REPORT OF THE SURGERY BRANCH  
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE  
OCTOBER 1, 1987 THRU SEPTEMBER 30, 1988

The clinical and laboratory research performed by the Surgical Branch is focused on elucidating solutions to long-standing clinical problems in cardiac surgery in the following areas: prosthetic heart valves, hypertrophic cardiomyopathy, metabolism during hypothermia with and without ischemia and/or cardiopulmonary bypass, augmentation of blood flow and power for ischemia cardiac failure and rejection phenomena association with cardiac transplantation.

METABOLIC STUDIES

Hypothermia continues to be a major component of clinical cardiac surgery. Blood flow, blood gas and pH management strategies during hypothermic cardiopulmonary bypass have developed empirically. Comparative physiological data have demonstrated differing behavior of acid-base regulation in ectothermic and hibernating animals. The purpose of the reported studies by Swain et al was to determine the effects of various pH management methods on heart, brain and total body metabolism under normothermia and varying degrees of hypothermia with and without cardiopulmonary bypass.

The first study used surface-induced hypothermia to avoid the complicating variable of cardiopulmonary bypass. Dogs (N=22) were cooled to 26°C and hemodynamic and biochemical variables were measured at 37, 32 and 26°C. Arterial blood pH was managed by a) maintaining a pH value of 7.4 (pH stat) or b) increasing arterial blood pH by 0.015 pH units for each degree of temperature decrease (alpha-stat). The mean arterial pressure and base excess calculations were different between the two groups. The alpha-stat group had a lower mean arterial pressure and a positive base excess whereas the pH-stat group had elevated mean arterial pressures and negative base excess. Systemic lactic acid concentrations and oxygen consumption were similar for both groups at all temperatures. The conclusions reached were that either pH management scheme provided adequate oxygen delivery and that alpha-stat management did not result in an increase in metabolism as proposed by others.

This initial study was then extended using NMR to determine if, under hypothermic conditions, the brain and heart independently autoregulated intracellular pH (pHi) or were dependent on blood pH. <sup>31</sup>P NMR spectroscopy was used to determine the pHi of brain and heart at 37°C and 26°C using sheep (N=15). Radiofrequency coils were placed over either the heart or the brain. The animals were randomly assigned to either alpha or pH stat management during hypothermia. The results showed that the intracellular pH was 7.10 for both brain and heart at 37°C. At 26°C, with an arterial blood pH of 7.4 the pHi of both organs remained at 7.10. In contrast, when the arterial blood pH was 7.58, the intracellular pH of both organs increased to 7.30. This study demonstrated that there was no evidence that heterotherms (i.e. humans) had a proton pump mechanism like that found in hibernators to maintain a



constant pHi. This study was then extended to include cardiopulmonary bypass with a further decrease in temperatures to 20°C (N=11). The alpha-stat scheme of pH management was used. No change in high energy phosphates were detected. The extracellular and intracellular pH followed the rise in blood pH.

Systemic deep hypothermia (14°C) and circulatory arrest are currently used to facilitate intracardiac repair in infants, aortic arch replacement and certain neurosurgical operations. Clinical observations have shown that the maximal duration of circulatory arrest was 45-60 minutes after which neurologic damage was found in the post-operative interval. The hypothesis tested in the next series of experiments was that intermittent low volume perfusion of the brain with a crystalloid solution similar to that used for myocardial preservation (cerebroplegia) would preserve high energy phosphate stores and intracellular pH of the brain during hypothermic hemodilution circulatory arrest. The 4.5 Tesla 30cm bore magnet was used during continuous spectra collection. The pre-arrest values for ATP, phosphocreatine and pH were similar in the group that received no perfusion (N=8) and that receiving cerebroplegia (N=7). The latter group maintain higher values of ATP, PCR and pHi during the arrest interval and during the initial 30-60 minutes of reperfusion. The EEG activity returned to the control waveform within 30 minutes in the treated animals versus 120 minutes in the non-treated animals. These data demonstrated that protection of the brain can be achieved in biochemical and electrical terms. Chronic studies to determine physiologic benefit are in progress.

A clinical study of thyroid hormone concentrations before, during and after cardiac surgery was completed. The hypothesis tested was that thyroid hormone concentrations were flow mode dependent. Two groups of similar patients had either pulsatile flow or mean flow perfusion under hypothermic hemodilutional conditions. The data demonstrated virtually no differences between the two flow states except for a transient rise in TSH levels and low concentrations of albumin in the post operative interval for the pulsatile group. The etiology or significance of the first observation is unknown. The second finding was probably a reflection of fluid management differences in the two groups. A third finding that may have clinical significance was that total T<sub>3</sub> remained at only 20% of preoperative values for the first 24 hours after operation for both groups of patients. The importance of this finding was that if adequate concentrations of triiodothyroxine are fundamental to cardiac function after ischemia and reperfusion, administration of this compound may be preferable to catecholamine administration for treatment of low cardiac output states.



## OBSTRUCTIVE HCM

Surgical treatments of the obstructive forms of hypertrophic cardiomyopathy (HCM) also known as idiopathic hypertrophic subaortic stenosis (IHSS) continue to be of major interest to the Surgery Branch. Two prospective clinical studies continue, a new one was initiated and three retrospective reviews were completed. Patients with the classic obstructive forms of this genetic disease continue to be treated with the operation developed by Morrow nearly thirty years ago. This operation (LVMM) involves removal of a rectangular block of myocardium from the left side of the hypertrophic septum to obtain a greater cross sectional area for blood flow through the left ventricular outflow tract. The procedure as refined by C.L. McIntosh, M.D. now carries a low mortality rate of 2-3%. It predictably relieves symptoms and markedly decreases the pressure gradients between the apex of the left ventricle and the aorta. An alternative to the standard operation was mitral valve replacement (MVR) which had previously been shown by others to abolish or reduce the gradients and relieve symptoms. A program of intraoperative echocardiography and selection of patients for MVR based on septal morphology and thickness was begun six years ago. The results show that the hospital mortality rate was approximately 5-6% and symptoms and gradients are abolished or greatly relieved. Perforation of thin septums was avoided by this operation. Long term followup with comparisons between the two groups of patients continues.

The mechanism(s) of obstruction in patients with HCM continues to be debated. It is the belief of the Surgery and Cardiology Branches that true mechanical impedance exists in the left ventricular outflow tract of hypertensive left ventricles of HCM patients. To test this hypothesis, simultaneous aortic, left ventricular, pulmonary artery and right atrial pressures and phasic aortic flow were measured simultaneously in patients requiring LVMM. Additionally, intraoperative echocardiographic studies were made with various modes (2-D, M., color, pulsed and continuous wave doppler). The data analysis includes detailed correlation of phasic hemodynamic events and intracardiac motion of the ventricular septum and anterior leaflet of the mitral valve before and after septal myectomy. The preliminary data demonstrate that high fidelity imaging and hemodynamic phasic data can be obtained simultaneously and that true mechanical impedance does exist.

Three retrospective reviews were completed. These examined outcome in patients with HCM and coronary artery disease, the effect of the Morrow operation on concurrent mitral regurgitation and the incidence of introgenic interventricular septal defect after septal myectomy. The analyses of the data showed that 1) the coexistence of coronary artery is a strong independent variable relative to perioperative mortality 2) the major contributor to the increased mortality rate in this subset of HCM patients was the intraoperative creation of VSD and 3) the major risk of VSD only occurred in patients with basal septal thickness of < 20mm. This review suggested that improved survival would be obtained in patients with coronary disease and thin septa by using a mitral valve replacement procedure instead of septal myectomy.



The effect of operation on the degree of mitral regurgitation was examined in patients with obstructive HCM with and without coronary artery disease. The results demonstrated that one half of those free of coronary disease had a decrease in mitral regurgitation while only one quarter of those with co-existent disease had a similar result. The interpretation of these findings was that coronary artery disease imposed additional papillary muscle dysfunction as a cause of mitral regurgitation which was not corrected by operation and that the abnormal left ventricular geometry associated with HCM was a major cause of mitral regurgitation. Relief of symptoms and abnormal gradients were obtained in all patients.

#### PROSTHETIC HEART VALVES

The effects of various devices used for palliative surgical treatment of valvular heart disease have been of intense interest to the Surgery Branch for nearly three decades. The continuing animal studies by the team headed by Michael Jones, M.D. accomplished substantial progress in the reporting interval. Studies of eight types of anticalcification treatments to valvular bioprostheses were contrasted to seven control groups. These data demonstrated that twenty weeks after implantation in the mitral position in juvenile sheep, the porcine aortic bioprosthesis treated with Polysorbate-80 had nearly twelve fold less calcium per gram of leaflet tissue than controls. Triton X-100 with N-lauryl sarcosine and sodium dodecyl sulfate treatments also decreased calcium accumulation within the leaflet tissue by eight and four fold, respectively. None of the treatments studied had significant effects on calcium deposition in bovine pericardial bioprostheses. However, ultrastructural microscopic studies showed that at least one of the antiminerallization processes resulted in structural alterations of collagen which could result in early leaflet fatigue. Complete hemodynamic studies have been performed immediately after implantation and at sacrifice. The first set of determinations characterized the devices for a constant size. Twenty-nine types of devices have been studied to date (8 bovine pericardial valves, 16 porcine aortic valves, 3 mechanical valves and 2 types of anuloplasty rings). At mean flows of  $3.0 \pm 0.1$  l/min and heart rates of  $110 \pm 10$  beats/min the pressure drop across these devices ranged from  $8.3 \pm 0.2$  to  $3.3 \pm 0.6$  mm Hg for porcine aortic valves to anuloplasty rings, respectively. All determinations were made immediately after implantation. These data represent the largest experimental experience known.

Ultrasonic studies were conducted using a color-encoded, two-dimensional Doppler device to determine velocity and turbulence relationships for prosthetic heart valves, anuloplasty rings and normal mitral valves. The studies of normal mitral valves, the first to systematically delineate in vivo velocity, flow and turbulence events, demonstrated that flow through the mitral valve and the closure events were very complex in contrast to conventional teachings. These should provide insight into the complexities of intracardiac physiologic events produced by prosthetic valvular devices. Pathologic studies of degenerated bioprostheses and healing patterns continue. Eight hearts were found to have ventricular damage associated with prosthetic valve implantation. The mechanisms have been determined and the results





published. Investigations continue on the mechanisms by which biologic tissue and synthetic biomaterials calcify and/or degenerate when placed inside the heart and are cyclically stressed.

In vitro characterization of prosthetic valves is an important compliment to the in vivo studies. A complex, highly instrumented pulse duplicator sytem has been used to charaterize systolic and diastolic function of 18 mechanical valves, 33 new bioprostheses and 12 clinically explanted bioprosthetic aortic prostheses. Peak pressure differences across the protheses were measured in lcm increments 1 to 6cm distal to the valve annulus. Simultaneous maximal velocities were obtained with continuous wave doppler ultrasound. The latter data were transformed to pressure differences by the modified Bervoulli equation. The data demonstrated that linear correlations for all valves were obtained with R values of 0.95 - 0.99, the slopes were valve type and orientation dependent. These data are clinically important because decisions concerning reoperation for stenotic prostheses are now currently made solely or in part from non invasive ultrasonic data. Major errors could be made.

The role of the chordal structures of the mitral valve in left ventricular function is under study. Preliminary data were obtained two years ago in sheep indicating a minimal role in preservation of function of the chordal apparatus in the presence of mitral regurgitation and use of mitral valve replacement. The hypotheses was that the mitral apparatus should be left intact during mitral valve replacment for severe mitral regurgitation because continuity between the papillary muscles and the mitral annulus prevented further ventricular dilatation and decreased function. To further test this hypothesis a colony of young sheep with severe mitral regurgitaion has been established and valve replacement with and without chordal retention is in progress.

A clinical study using computer coupled phonocardiography has been used to study the sounds of heart valves in more than 400 patients. The hypothesis tested was that each type and size of valve had a normal sound signature and alterations of that signature were reflections of changes in the biomaterials and/or impedence to opening and/or closing by thrombus. Energy in terms of dB as a function of frequency for the four major intervals of valve function were analyzed. All valves removed from patients for clinical dysfunction had grossly abnormal closure signatures. An aging characteristic has been detected in aortic bioprostheses but not for mechanical types. Signature abnormalities before clinical symptoms develop have been found. Correlations of early dysfunction by sound analyses and hemodynamic angiographic data have not been obtained to date. These data will be obtained from serial measurements of valve sounds and hemodynamic variables in the same patients over several years.



## MYOCARDIAL PRESERVATION

Five studies were initiated concerning new methods for increasing protection of the myocardium during global ischemia. Three studies used isolated rat heart preparations with a working left ventricle. 85 hearts were used to study a synthesized prostaglandin trimer which had been used to protect the brain from hypoxemia in rodents. Although the percentage of hearts that recovered from a 27 minute normothermic ischemic interval were different between control and those receiving the drug, the post-ischemic hemodynamic variables between groups were not different. It was concluded that this compound had little if any myocardial protective effect. A second study with similar methodology was performed using nicarpidine, a slow channel blocker and an analog of nifedipine. The drug is not light sensitive as is the parent compound, and has little effect on blood pressure. The data showed that there was a narrow dose response range at 37°C. The drug was highly efficacious for myocardial protection when given during a 27 minute ischemic interval at normothermia. At 10°C no protective effect was demonstrated although hypothermia was highly protective with 85-95% return of control values after 3.5 hours of ischemia. The studies continue.

Amiodarone has been found to have a toxic effect in patients who require cardiac surgery. Amiodarone toxicity was studied in normotensive and spontaneously hypertensive rats. The two groups were given chronic administration of the drug at clinically analogous doses for 3-9 months. Using a left working heart preparation, it was shown for both pressure groups of rats that amiodarone caused myocardial depression compared to age/sex matched controls. Drug treated animals had a decreased tolerance to ischemia and reperfusion. These data support the clinical observations.

The issue of tolerance of cyanotic myocardium to ischemia is important in neonatal and pediatric cardiac surgery. Clinical observations have shown that hearts of cyanotic babies tolerate ischemia poorly in contrast to those of non-cyanotic newborns. Accordingly, young dogs were made cyanotic and six months later had an open heart operation using cardiopulmonary bypass, hypothermia and global myocardial ischemia and reperfusion simulating the clinical setting. The preliminary data suggest that cyanotic hearts had decreased contractility and after the reperfusion interval profound cardiac failure occurred in 2-3 hours. In contrast, the control group showed a pattern of increased to stable contractility after cessation of cardiopulmonary bypass. If the large and distinct difference in post ischemia-reperfusion performance can be substantiated in more animals, various interventions directed initially at oxygen free radical capture will be undertaken.



## OTHER

A widely heralded method for augmentation of power for the failing myocardium was tested in dogs. A skeletal muscle (latissimus dorsi) was wrapped around the left ventricular mass and then chronically stimulated with an atrioventricular pacemaker. Muscle transformation was accomplished in all but one animal. Selective left main coronary artery infusion of lidocaine resulted in a 50% decrease in ventricular contractility and measurements of cardiac function were made with the pacemaker on and off to determine efficacy of the transformed skeletal muscle wrap. No power augmentation was demonstrated. Lidocaine had no effect on skeletal muscle function. It was concluded that the clinical operation as now performed has no experimental confirmation of efficacy. Further, a reversible predictable method for incurring left heart failure in dogs was developed.

Two projects have centered on problems of rejection. The first used isolated pig hearts to study the mechanisms of hyperacute rejection. A sophisticated extracorporeal circulation system employed blood and blood fractions from pigs, dogs, baboons, and humans. Hyperacute rejection was obtained consistently with high concentrations of human plasma and with whole blood from dogs. No rejection was obtained with autologous pig or whole baboon blood. The interpretation was that pre-formed natural antibodies present in plasma, but not on red cells was the etiologic factor. Work continues to isolate the antibodies responsible. The significance is that the component(s) of plasma responsible for hyperacute rejection, once discovered, can be determined preoperatively and loss of a transplanted heart can be avoided.

The second transplant study tested the hypothesis that the IL-2 receptor of T-cells activated by interaction with foreign antigens was a major contributor to chronic rejection of transplanted solid organs. Anti-tac, a monoclonal antibody to the interleukin-2 receptor was used alone, conjugated to pseudomonal exotoxin and chelated with 90 yttrium a beta emitter. Xenografts of cynomolgus hearts into the necks of rhesus monkeys were used. Animals without immunosuppression rejected the xenograft in  $\leq 7$  days as did those receiving the unmodified anti-tac. Pseudomonal exotoxin anti-tac was toxic in five of seven monkeys. Five animals which received a total dose of 16 - 20 mci of anti-tac + 90 yttrium divided in four doses survived  $38 \pm 5$  days with no significant toxicity. No isotope was given after the 14th postoperative day. The significance of these data are that a receptor specific immunosuppressor system has shown efficacy which may obviate the present suppression therapies and complications therefrom. Further, the system could ultimately lead to clinical use of xenografts.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02714-08 SU

## PERIOD COVERED

October 1, 1987 thru September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Evaluations of Prosthetic Cardiac Valves: In Vivo Experimental Studies

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Michael Jones, M.D., Senior Investigator, &amp; Surgeon, Surgery Branch, NHLBI

Elling E. Eidbo, B.A., Research Assistant, Surgery Branch, NHLBI

Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

## COOPERATING UNITS (if any)

Cardiovascular Fluid Dynamics Laboratory, Georgia Institute of Technology

Ultrastructural Section, Pathology Branch, NHLBI

Center for Devices and Radiological Health, Food and Drug Administration

## LAB/BRANCH

Surgery Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

7.5

## PROFESSIONAL:

2.5

## OTHER:

5.0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

The studies of this project include those of the ongoing development and application of an in vivo experimental animal model system using juvenile sheep for evaluating cardiac valves. The studies are multidisciplinary in nature, using investigative techniques from the fields of surgery, physiology, pathology, engineering, and technology development. Major emphases during the year have been: 1) studies of the efficacy of preimplantation treatment regimens upon ameliorating the calcification of bioprosthetic valves; 2) definitions of the velocity, flow and turbulence profiles produced by prosthetic valves using ultrasound techniques; 3) comparisons of fluid dynamic data obtained from the in vivo (animal) studies and those obtained from in vitro studies; 4) comparisons of old generation (introduced prior to 1975) versus new generation - valves; 5) comparative hemodynamic/fluid mechanic studies of prosthetic valves used clinically or undergoing preclinical evaluations; 6) continuing characterization of the morphologic pathologic alterations occurring in bioprosthetic valves; and 7) refinements of operative and postoperative management techniques.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02731-06 SU

## PERIOD COVERED

October 1, 1987 through September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Operative Assessment and Results of Left Ventriculomyotomy and Myectomy

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Charles L. McIntosh, M.D., Ph.D., Senior Surgeon, Surgery Branch, NHLBI

Barry Maron, M.D., Senior Investigator, Head, Echo Lab, Cardiology Branch, NHLBI

## COOPERATING UNITS (if any)

Cardiology Branch, NHLBI

## LAB/BRANCH

Surgery Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.5

## PROFESSIONAL:

1.0

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A standard left ventriculomyotomy and myectomy (LVMM) has been performed for relief of left ventricular outflow tract obstruction secondary to idiopathic hypertrophic subaortic stenosis (IHSS) in 463 patients. This report summarizes 133 patients undergoing an LVM&M since January 1982. An attempt has been made to define criteria for choice of operation, LVM&M vs. mitral valve replacement (MVR) based upon septal thickness, distribution of hypertrophy, level of systolic anterior motion (SAM), contact of septum, and concomitant coronary artery disease. Intraoperative 2-D and M-mode echos have been performed on a number of these patients providing precise data utilized intraoperatively. Patients with concomitant CAD are at greater risk for an iatrogenic VSD creation which may be avoided by a modified LVM&M or MVR. Operative mortality is 3.0% and late mortality 3.0%. Results are presented based on preoperative resting gradients < 50 mm Hg and > 50 mm Hg. Postoperative hemodynamic studies reveal good relief of resting gradient in most patients but significant provokable gradients remain in some patients. Three patients have demonstrated significant RVOT obstruction (> 50 mm Hg) and underwent concomitant LVM&M and resection of RVOT obstruction. Two patients developed late VSD's; one required closure for a QP:QS of 2.6:1. Reoperation has been performed in some patients with persistent symptoms and gradients. Medical therapy is continued in patients with significant gradients regardless of symptomatic status.

805



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02733-05 .SU

## PERIOD COVERED

October 1, 1987 thru September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mitral Valve Replacement in Selected Patients Having IHSSPRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)  
Charles L. McIntosh, M.D., Ph.D., Senior Surgeon, Surgery Branch, NHLBI

Barry Marron, M.D., Senior Investigator, Head, Echo Lab, Cardiology Branch, NHLBI

## COOPERATING UNITS (if any)

Cardiology Branch

## LAB/BRANCH

Surgery Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung and Blood Institute, NIH Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.5

## PROFESSIONAL:

1.0

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects☐ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Mitral valve replacement (MVR) has been performed on 48 patients as primary or secondary treatment of severely symptomatic patients with resting and/or provokable pressure gradients across the left ventricular outflow tract (LVOT) secondary to idiopathic hypertrophic subaortic stenosis (IHSS). Indications for MVR include: 1) septal thickness < 18 mm; 2) persistent LVOT obstruction after a prior adequate left ventriculomyotomy and myectomy (LVM&M); 3) atypical septal morphology; and 4) severe mitral regurgitation secondary to ruptured chordae tendinae or papillary muscle. Intraoperative echocardiography has provided definition of septal morphology allowing selection for MVR. There have been 3 (6.2%) perioperative deaths, one a result of hepatic failure one suspected to be caused by prosthetic valve malfunction, and 2 secondary to infection. Three patients (6.9%) died after hospital discharge, two suddenly and one of congestive heart and respiratory failure. One patient had a late central embolus. Symptomatic improvement to NYHA functional class I or II has occurred in 86% of 36 patients returning for postoperative evaluation. Excellent relief of both resting and provokable gradients has been demonstrated. Four patients continue to be symptomatic (FC III) and have been shown to have abnormal coronary vascular resistances with no reserve indicating the presence of severe small vessel disease. Thus relief of LVOT obstruction does not always relieve symptoms of chest pain and fatigue. Long-term follow-up will be necessary to assess late mortality and morbidity which will be compared to the well-known results of LVM&M used for palliation in IHSS for the past 26 years.

fuf



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02740-05-SU

## PERIOD COVERED

October 1, 1987 Thru September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Coronary Vascular Resistance and Cardiac Metabolism in the Postoperative Period

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Lester C. Permut, M.D., Principal Investigator, Surgery Branch, NHLBI

Ira L. Siegman, M.D., Medical Staff Fellow, Surgery Branch, NHLBI

Julie A. Swain, M.D., Senior Investigator and Surgeon, Surgery Branch, NHLBI

Thomas J. McDonald Jr., B.S., Research Technician, Surgery Branch, NHLBI

Joseph E. Flack, M.D., Senior Staff Fellow, Surgery Branch, NHLBI

Chahine Yamine, M.D.

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Surgery Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

5.0

## PROFESSIONAL:

5.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The hypotheses tested in this study are: 1) Systemic hypertension in the immediate postoperative period is detrimental to coronary artery blood flow and to myocardial metabolism, 2) The ideal level of systemic arterial pressure with regard to myocardial metabolism can be determined using a thermodilution flow catheter placed in the great cardiac vein, 3) Mode of pacing may effect myocardial metabolism. Prior to operation the coronary sinus and great cardiac vein are cannulated with a Thermister catheter. Serial determinations of coronary flow, lactic acid concentrations and a host of hemodynamic variables are made before and after coronary artery bypass. In the postoperative interval, systemic blood pressure is altered to assess myocardial metabolism at various levels. Various modes of pacing are used to determine the effects on myocardial metabolism. These studies should determine the efficacy of blood pressure control and pacing mode on myocardial metabolism in the immediate postoperative interval after coronary artery bypass and other procedures.

811



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02774-03 SU

## PERIOD COVERED

October 1, 1987 thru September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

In vitro Assessment of Noninvasive Methods for Evaluation of Prosthetic Heart Valves

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Edward P. Nast, M.D., Principal Investigator, Surgery Branch, NHLBI

Sandy F.C. Stewart, Ph.D., Senior Staff Fellow, Surgery Branch, NHLBI

Francisco A. Arabia, M.D., Clinical Associate, Surgery Branch, NHLBI

Thomas L. Talbot, M.M.E., Staff Engineer, Biomedical Engineering and Instrumentation Branch, Division of Research Services

Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

## COOPERATING UNITS (if any)

BEIB, ORS

## LAB/BRANCH

Surgery Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2.0

## PROFESSIONAL:

2.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The hypotheses tested in these in vitro studies were: a) continuous wave Doppler CWD provides accurate assessment of pressure differences across prosthetic valves as a function of flow, b) CWD and phonocardiography are accurate methods for noninvasive in vitro evaluation of the hydrodynamic performance of normal and explanted prosthetic heart valves and c) these techniques also detect early prosthetic valve failure and are accurate noninvasive procedures for serial evaluation of prosthetic valve function. A physiologic pulse duplicator with pressure and electromagnetic flow transducers, and IBM AT microprocessor, and IREX Meridian ultrasonic system, and an International Acoustics, Inc. phonocardiography amplifier system were used. Eighteen new mechanical, thirty-three new bioprosthetic, and twelve explanted bioprosthetic aortic prostheses were studied. Peak pressure differences were measured at 1 cm increments from 1 to 6 cm distal to the valve annulus for determination of maximal peak pressure differences. Simultaneous maximal velocities were obtained with CWD and transformed to pressure differences with the modified Bernoulli equation. Complete evaluation of the data for all valves studied is currently in progress.

813





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02776-02 SU

## PERIOD COVERED

October 1, 1987 thru September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Effect of Left Ventricular Septal Myectomy on Concurrent Mitral Regurgitation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

Matthew M. Cooper, M.D., Principal Investigator, Surgery Branch, NHLBI

Eben Tucker, M.D., Cardiology Consultant, Cardiology Branch, NHLBI

Charles L. McIntosh, M.D., Ph.D., Senior Surgeron, Surgery Branch, NHLBI

Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

## COOPERATING UNITS (if any)

Cardiology Branch, NHLBI

## LAB/BRANCH

Surgery Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung and Blood Institutes, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2

## PROFESSIONAL:

2

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The hypothesis tested was that left ventricular septal myectomy improved coexisting mitral regurgitation. The pre- and postoperative cine left ventriculograms of 39 patients were compared. The mean difference in time between studies was 15 months. Approximately one half of the patients had a reduction in mitral regurgitation. There was no association between relief of obstructive pressure gradient at rest or with provocation or symptoms and improvement in mitral regurgitation.

8/6



## NOTICE OF INTRAMURAL RESEARCH PROJECT

201 HL 02777-02 SU

## PERIOD COVERED

October 1, 1987 thru September 30, 1988

## TITLE OF PROJECT (80 characters or less. The title is on one line between the borders)

The use of monoclonal anti-Tac antibody as immunosuppression for cardiac xenografts

PRINCIPAL INVESTIGATOR and other professional personnel below the Principal Investigator, state the address and phone number  
Robert C. Robbins, M.D., Principal Investigator, Surgery Branch, NCI

Thomas A. Waldman, M.D., Chief, Immunology Branch, NCI

Otto A. Gansow, Ph.D., Head, Inorganic and Radioimmunochemistry Section, Radiation Oncology Branch, NCI

Richard E. Clark, M.D., Chief, Surgery Branch, NCI

## COOPERATING UNITS (If any)

Immunology Branch, NCI

Inorganic and Radioimmunochemistry Section, NCI

LAB/BRANCH

Surgery Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

## PROFESSIONAL:

## OTHER:

3

1

1

## CHECK APPROPRIATE BOXES

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard language form. Do not exceed the space provided)

This study is designed to evaluate the effectiveness of three forms of anti-Tac, a monoclonal antibody that recognizes the interleukin-2 (IL-2) receptor as sole immunosuppressive therapy in a model of heterotopic primate cardiac xenografting. The IL-2 receptor is not expressed on resting cells but is expressed on T-cells activated by interaction with foreign transplantation antigens. Unconjugated anti-Tac and anti-Tac chelated to pseudomonal exotoxin and to <sup>90</sup>Yttrium (Y-90), a beta emitting isotope, are evaluated for efficacy and safety. Analysis of graft survival, modification of the composition of specific T-cell phenotypes, and toxicity were performed by comparing animals receiving no immunosuppression (n=3) with those receiving the various forms of anti-Tac (N=15). Five animals receiving anti-Tac only failed to have increased survival. Five of seven Rhesus receiving the exotoxin died of toxicity. Five animals received adequate doses of Y-Tac for 14 days in total doses of 14-18 mCi with a prolongation of graft survival of 38 ± 5 days. The evaluation of possible enhanced efficacy of the monoclonal chelates over the unmodified form as well as toxicity accrued will have direct relevance to human use of such agents in organ transplantation and in the treatment of adult T-cell leukemia, a leukemia expressing the Tac antigen.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02778-02 .SU

## PERIOD COVERED

October 1, 1987 thru September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Effect of Colloid Versus Crystalloid Fluid Administration on Lung Water during CPBP

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Marc E. Mitchell, M.D., Principal Investigator, Surgery Branch, NHLBI

Julie A. Swain, M.D., Senior Surgeon, Surgery Branch, NHLBI

Charles L. McIntosh, M.D., Senior Surgeon, Surgery Branch, NHLBI

Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Surgery Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2.0

## PROFESSIONAL:

2.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects☐ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Forty adult patients undergoing elective surgical procedures requiring cardiopulmonary bypass will be studied in the perioperative period including the 24 hours immediately following operation. The hypotheses to be tested are: 1) measures of extravascular lung water and colloid osmotic pressure can be used to predict the postoperative clinical respiratory status of patients; and 2) the use of a colloid rather than a crystalloid solution for perioperative fluid resuscitation affects postoperative respiratory function and the need for respiratory support, hemodynamic variables, and cost. Possible adverse effects associated with either fluid resuscitation regimen will be determined.

f22



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02779-02 SU

## PERIOD COVERED

October 1, 1987 thru September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Effect of Hypothermia on Intracellular pH: A <sup>31</sup>P NMR Study.

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Julie A. Swain, M.D., Senior Investigator, Surgery Branch, NHLBI

Robert Balaban, Ph.D., Chief, Laboratory of Cellular Energetics, NHLBI  
Thomas J. McDonald Jr., B.S., Research Assistant, Surgery Branch, NHLBI  
Robert C. Robbins, M.D., Medical Staff Fellow, Surgery Branch, NHLBI

## COOPERATING UNITS (if any)

Laboratory of Cellular Energetics

## LAB/BRANCH

Surgery Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2.0

## PROFESSIONAL:

1.5

## OTHER:

.5

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Laboratory studies have shown that the blood pH during hypothermia profoundly affects the heart and other organs. One of two pH schemes is commonly used to manage blood pH during hypothermia. The alpha-stat scheme is the method by which ectotherms (or cold-blooded animals) manage pH. In this strategy, the pH of blood is increased 0.015 pH units for every one degree decrease in temperature. When the pH is kept constant during hypothermia (7.4 in man), the pH-stat strategy is employed. This is the strategy employed by hibernators during hypothermia. Comparative physiologists have found that the intracellular pH of all organs in the ectotherm parallels the blood pH. This is different from the intracellular pH in hibernators where most organs, such as the brain and skeletal muscle, maintain a constant pH, while essential organs such as the heart and liver have an intracellular pH that follows the ectothermic scheme. The intracellular pH is important to the function of most organ systems.

The hypothesis to be tested in the present investigation was that brain and heart tissue pH in heterothermic animals during hypothermia is determined by blood pH. The experimental model consisted of anesthetized sheep wrapped in cooling blankets with a radiofrequency coil placed over either the heart or skull. <sup>31</sup>P NMR spectroscopy was performed to assess the intracellular pH at 37°C and during cooling to 26°C.

The results show that the intracellular pH of the brain and the heart at 37°C was approximately 7.10. During hypothermia the intracellular pH of both of these tissues parallels the blood pH. That is, during pH stat management, the intracellular pH is constant, and during alpha-stat management, the intracellular pH increases with hypothermia. This has important implications for organ function during hypothermic cardiac surgical procedures in man.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02780-02 SU

## PERIOD COVERED

October 1, 1987 thru September, 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Effect of Hypothermia on Myocardial Tissue pH during Cardiopulmonary Bypass.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Robert C. Robbins, M.D., Principle Investigator, Surgery Branch, NHLBI

Julie A. Swain, M.D., Surgeon and Senior Investigator, Surgery Branch, NHLBI

Thomas J. McDonald, B.S., Technician, Surgery Branch, NHLBI

Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Surgery Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2.0

## PROFESSIONAL:

2.0

## OTHER:

1.0

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The effect of hypothermia on intracellular myocardial pH in humans is unknown. The acid-base status of blood during systemic hypothermia theoretically should have profound effects on myocardial tissue pH. Variations in myocardial tissue pH affect enzyme function and the ability of the organ to withstand ischemia. The purpose of this project is to determine whether myocardial pH varies with changes in blood pH.

The NIH tissue pH probe has been inserted in the myocardium of 16 patients during hypothermic cardiopulmonary bypass. The acid-base scheme followed was either that of alpha-stat (N=3) or pH-stat (N=11) and the tissue pH was compared to that of the myocardium using these two pH management schemes.

Two patients had inadequate data. The preliminary results show that the changes in myocardial pH parallel those of the blood pH. The study is to continue.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02781-01 SU

## PERIOD COVERED

October 1, 1987 thru September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cerebral Protection

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Robert C. Robbins, M.D., Principal Investigator, Surgery Branch, NHLBI

Julie A. Swain, M.D., Surgeon and Senior Investigator, Surgery Branch, NHLBI

Robert Balaban, Ph.D., Chief, Laboratory of Cellular Energetics, NHLBI

## COOPERATING UNITS (if any)

Laboratory of Cellular Energetics, NHLBI

## LAB/BRANCH

Surgery Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This study was designed to evaluate the use of a cold crystalloid intermittent cerebral perfusion, cerebroplegia, for the preservation of cerebral high energy phosphates during a two hour period of hypothermic total circulatory arrest and reperfusion. NMR spectroscopy was utilized to record changes in cerebral ATP, PCR, and intracellular pH. Cardiopulmonary bypass (CPB) was employed to achieve hemodynamic stability during cooling and reperfusion. Sheep (20-35 kg) were divided into 2 groups. Group I received no cerebroplegia and served as the control group. Group II received cerebroplegia. No significant differences were present between the groups prior to arrest. ATP, PCR, and pH were maintained higher in the Group II animals for all points during the arrest period and until 60 minutes following reperfusion. ( $p < .05$ ) For the remaining three hours of reperfusion no differences were noted. EEG activity returned after 30 minutes of reperfusion in Group II compared to 120 minutes in Group I. ( $p < .05$ ) We have developed a system for the NMR evaluation of cerebral high energy phosphate changes during hypothermic total circulatory arrest and reperfusion using CPB in large animals. Cerebral high energy phosphates and pH are maintained during the arrest period with the administration of cerebroplegia. The rapid return of the EEG signal in the cerebroplegia animals suggests protection was achieved, however further studies must be done to assess the benefits of cerebroplegia in animals that are recovered for functional neurological evaluation.

833



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02782-01 SU

## PERIOD COVERED

October 1, 1987 thru September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Amelioration of Hyperacute Rejection

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Robert C. Robbins, M.D., Principal Investigator, Surgery Branch, NHLBI

Marc E. Mitchell, M.D., Medical Staff Fellow, Surgery Branch, NHLBI

David Sachs, M.D., Senior Investigator, Immunology Branch, Division of Cancer Biology and Diagnosis, NCI

Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

## COOPERATING UNITS (if any)

Immunology Branch, DCBD, NCI

## LAB/BRANCH

Surgery Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The use of large domestic animals as heart donors represents a potential solution to the current human donor shortage. Hyperacute graft rejection (HAR) remains a major barrier to xenotransplantation. The purpose of this study was to employ an ex-vivo preparation for the evaluation of HAR in discordant cardiac xenografts. Freshly excised hearts from thirty-nine pigs (10-37kg) were perfused at 37 degrees centigrade via the aorta in a retrograde fashion. The hearts were allowed to function in a non-working mode for four hours or until the rejection process resulted in irreversible cardiac dysfunction. The perfusate consisted of Krebs-Henseleit bicarbonate buffer in addition to fresh whole autologous pig blood(n=4) (Group A), dog blood(n=3) (Group B), baboon blood(n=5) (Group C), human PRBCs (n=2) (Group D), human blood and plasma (n=3) (Group E), human whole blood (n=10) (Group F), and human plasma(n=9) (Group G). HAR was uniform for Groups B, E, and G. No evidence of HAR was noted in Groups A, C, or D. Two of ten hearts demonstrated HAR in Group F. This perfusion circuit provides a means for the analysis of each component of human blood in order to define which components are required for HAR of discordant cardiac xenografts. The results indicate that the HAR of pig hearts perfused with human blood is mediated by some component found in the plasma, most probably pre-formed natural antibody. Studies are currently underway to evaluate which class of antibody mediates the rejection process.

f36



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02783-01 SU

## PERIOD COVERED

October 1, 1987 thru September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Effects of Chronic Amiodarone Administration on Cardiac Function

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Marc E. Mitchell, M.D., Principle Investigator, Surgery Branch, NHLBI

Robert C. Robbins, M.D., Medical Staff Fellow, Surgery Branch, NHLBI

John P. Kupferschmid, M.D., Medical Staff Fellow, Surgery Branch, NHLBI

Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Surgery Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Low cardiac output syndrome has been reported after cardiac surgery in patients who have received chronic amiodarone administration. To test for possible cardiac toxicity of amiodarone, the isolated working heart model was used.

Chronic administration of amiodarone resulted in decreased heart rate and contractility in normal rats. Spontaneously hypertensive rats which received the drug had decreased heart rate, aortic output, and coronary flow compared to age/sex matched controls prior to ischemia. The conclusions were that chronic administration of amiodarone to normal rats caused a significant decrease in post-ischemia performance. Hypertrophied rat hearts are similarly sensitive to the drug.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02784-01 SU

## PERIOD COVERED

October 1, 1987 thru September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Myocardial Preservation with Nicarpidine

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Marc E. Mitchell, M.D., Principal Investigator, Surgery Branch, NHLBI

Michael D. Crittenden, M.D., Senior Fellow, Surgery Branch, NHLBI

Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Surgery Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NHLBI

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

To date approximately 50 rat hearts have been given cardioplegia containing nicardipine. It appears that nicardipine has a beneficial effect in protecting myocardium during ischemia at 37.5°C, but not at 10°C. The optimal dose of nicardipine for myocardial preservation has not yet been determined. These results are preliminary, and approximately 50 more animals will be required to complete the protocol.

840



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 HL 02785-01 SU
PERIOD COVERED October 1, 1987 thru September 30, 1988		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Simultaneous Intraoperative Echo & Hemodynamic Evaluation of Obstructive HCM		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) Edward P. Nast, M.D., Principal Investigator, Surgery Branch, NHLBI  Barry J. Maron, M.D., Cardiologist, Cardiology Branch, NHLBI Lester C. Permut, M.D., Medical Staff Fellow, Surgery Branch, NHLBI Ira L. Siegman, M.D., Medical Staff Fellow, Surgery Branch, NHLBI Robert C. Robbins, M.D., Medical Staff Fellow, Surgery Branch, NHLBI Charles L. McIntosh, M.D., Surgeon and Senior Investigator, NHLBI Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI Christopher Stone, M.D., Medical Staff Fellow, Surgery Branch, NHLBI		
COOPERATING UNITS (if any)  Cardiology Branch LAB/BRANCH Surgery Branch SECTION		
INSTITUTE AND LOCATION National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS: 1.0	PROFESSIONAL: 1.0	OTHER:
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  The hypotheses to be tested in these intraoperative studies are: a) true obstruction to left ventricular outflow due to mechanical impedance exists in patients with hypertrophic cardiomyopathy (HCM) who have a subaortic pressure gradient and b) operative intervention to relieve this obstruction results in relief of the obstruction and elevated intraventricular systolic pressures, which are detrimental to left ventricular performance. Solid state pressure transducers, electromagnetic flow transducers, and a Hewlett-Packard ultrasonic system will be applied under direct exposure intraoperatively in consecutive obstructive HCM patients undergoing first-time operative intervention. Echocardiographic studies preoperatively, intraoperatively (pre-and post-procedure), and postoperatively will include 2-dimensional (2-D), M-mode, and color Doppler imaging, in addition to pulsed Doppler, and continuous wave Doppler (CWD). The intraoperative echocardiography will be obtained simultaneous with hemodynamic measurements of left ventricular outflow tract (LVOT) pressure gradient and aortic blood flow. The findings of this study are expected to provide evidence supporting the presence of true mechanical obstruction of the LVOT in patients with HCM. These data should support the increased use of surgical palliation in selected patients.		

842



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02786-01 SU

## PERIOD COVERED

October 1, 1987 thru September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Dynamic Cardiomyoplasty

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Lester C. Permut, M.D., Principle Investigator, Surgery Branch, NHLBI

Ira L. Siegman, M.D., Medical Staff Fellow, Surgery Branch, NHLBI

Julie A. Swain, M.D., Senior Surgeon and Investigator, Surgery Branch, NHLBI

Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Surgery Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2.0

## PROFESSIONAL:

2.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The hypotheses tested in this study were that 1) the use of chronically stimulated latissimus dorsi muscle can augment cardiac function in a failing canine heart, and 2) intracoronary lidocaine infusion can produce a model of left ventricular dysfunction. The study used dogs that had latissimus dorsi muscle wraps of the left ventricle and were stimulated by a pacemaker synchronously with the heart beat. A method of producing profound depression of ventricular contractability was used and found to be reproducible and reversible. The augmentation of cardiac power by the muscle was determined by serial hemodynamic and angiographic measurements with and without flap stimulation with and without coronary lidocaine infusion. The results showed no augmentation of left ventricular power under any condition. These data show that the experimental operation as now performed in patients lacks laboratory confirmation and a reassessment of efficacy is necessary.

845



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02787-01 SU

## PERIOD COVERED

October 1, 1987 thru September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Ventricular Septal Defect after Left Ventricular Myotomy-Myectomy

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Lester C. Permut, M.D., Principal Investigator, Surgery Branch, NHLBI

Ira L. Siegman, M.D., Medical Staff Fellow, Surgery Branch, NHLBI

Charles L. McIntosh, M.D., Senior Surgeon and Investigator, Surgery Branch, NHLBI

Margaret Wu, Ph.D., Biostatistician, Biostatistics Research Branch, NHLBI

Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

## COOPERATING UNITS (if any)

Biostatistics Research Branch

## LAB/BRANCH

Surgery Branch, NHLBI

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The clinical impression that patients with hypertrophic cardiomyopathy and coronary artery disease had higher mortality rates and different septal morphology than HCM patients without coronary artery disease was tested by a review of the NHLBI experience.

A retrospective study of all patients undergoing left ventricular myotomy-myectomy (LVMM) performed as a first-time procedure with or without concomitant coronary artery bypass grafting was undertaken. A total of 415 patients were included. In each patient, age, sex, presence of coronary artery disease, occurrence of ventricular septal defect, hemodynamic data, and echocardiographic data were recorded. Coronary artery disease was considered to be present if 1) >75% stenosis of 1 or more extramural coronary artery was noted by coronary angiography, 2) >75% stenosis of 1 or more extramural coronary artery was noted at autopsy within 6 months of operation. Coronary artery disease was considered to be absent if 1) normal coronary angiography was documented immediately before or any time after operation, 2) no coronary artery disease could be documented at autopsy, 3) patients were 30 years of age or less. All patients not meeting these criteria were considered to have indeterminate coronary artery disease. Multivariate analysis of the data demonstrated that coronary artery disease was a significant independent predictor of VSD after LVMM. Interestingly, coronary artery bypass increased the likelihood of VSD. Further study of pathologic specimens and results of operative and nonoperative management will be described.

249





## NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02788-01 SU

## PERIOD COVERED

October 1, 1987 thru September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Surgical Treatment of Hypertrophic Cardiomyopathy and Coronary Artery Disease

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Ira L. Siegman, M.D., Principal Investigator, Surgery Branch, NHLBI

Barry J. Maron, M.D., Senior Investigator, Cardiology Branch, NHLBI

Lester C. Permut, M.D., Medical Staff Fellow, Surgery Branch, NHLBI

Charles L. McIntosh, M.D., Surgeon and Senior Investigator, Surgery Branch, NHLBI

Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

## COOPERATING UNITS (if any)

Cardiology Branch

## LAB/BRANCH

Surgery Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2.0

## PROFESSIONAL:

2.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects☐ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

A retrospective study was carried out in which all patients operated on simultaneously for hypertrophic cardiomyopathy and coexistent coronary artery disease were included. 28 patients were identified, four undergoing mitral valve replacement (MVR) and coronary bypass grafting (CABG) and 24 undergoing left ventricular myotomy-myectomy (LVMM) and CABG. Mean age at operation was 59 years (range 42 - 74 yrs). Five patients (18%) died as a result of operation, while 3 died after the immediate postoperative period. Mean period of follow-up for the 20 patients currently alive was 4.8 years (range 4 months to 10.8 yrs). 19 patients experienced substantial long-term symptomatic improvement. Long-term relief of chest pain was noted in 17 of the 20 surviving patients. Significant reduction in basal and provokable gradients were demonstrated in this group of patients after operation ( $P < 0.001$ ). 5 of the 24 patients undergoing LVMM incurred iatrogenic ventricular septal defect (VSD). The rate of this complication was higher in this group than that reported in all patients undergoing LVMM. This occurred primarily in patients with septa  $< 20$  mm as measured by echocardiography. VSD was responsible in large part for the relatively high mortality rate. It is suggested that MVR be considered as a preferable operation to LVMM in patients with coexistent CAD and relatively thin septa.

857



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02789-01 SU

## PERIOD COVERED

October 1, 1987 thru September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Physiology of Skeletal Muscle Transformation

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Ira L. Siegman, M.D., Principal Investigator, Surgery Branch, NHLBI

Lester C. Permut, M.D., Medical Staff Fellow, Surgery Branch, NHLBI

Isabella Liang, Ph.D., Senior Staff Fellow, Surgery Branch, NHLBI

Julie A. Swain, M.D., Senior Investigator and Surgeon, Surgery Branch, NHLBI

Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Surgery Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The hypotheses tested in these studies were 1) Endothelial cell growth factor (ECGF) may play a role in the transformation of skeletal muscle by chronic stimulation from an easily fatigable state to one of fatigue-resistance. 2) Transformed skeletal muscle responds to catecholamines in a manner different to that of non-transformed skeletal muscle. Conditioned latissimus dorsi muscles of dogs were created using an implanted pacemaker. Serial biopsies of the stimulated and contralateral none stimulated muscles were performed for ECGF and histology. At sacrifice dose-response experiments to catecholamines will be conducted to determine if slow fiber transformation alters pharmacologic responses.

f53



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02790-01.SU

## PERIOD COVERED

October 1, 1987 thru September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Myocardial protection of cyanotic hearts.

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Joseph E. Flack, M.D., Principal Investigator, Surgery Branch, NHLBI

Isabella Liang, Ph.D., Senior Staff Fellow, Surgery Branch, NHLBI

Julie A. Swain, M.D., Senior Surgeon and Investigator, Surgery Branch, NHLBI

Victor Ferrans, M.D., Pathologist and Senior Investigator, Pathology Branch, NHLBI

Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

## COOPERATING UNITS (if any)

Pathology Branch, NHLBI

## LAB/BRANCH

Surgery Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

3

## PROFESSIONAL:

2

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The tolerance to global cardiac ischemia and reperfusion, necessary for repair of congenital defects in the young has been found to be poor in cyanotic patients compared to those without cyanosis. The purpose of these studies was to develop a colony of cyanotic young dogs to determine the effect of chronic hypoxenia on myocardial function and develop methods to improve tolerance to global ischemia.

Foxhound puppies underwent the greation of a right to left shunt between pulmonary artery and left atrium using absorbable sutures. This created a level of cyanosis which was found to remain constant despite a 3 to 4 fold increase of weight over a 6 month adaptive period.

Currently, we are involved in the second phase of this study. This involves acute of these animals at the end of this 6 month period and measurement of left ventricular function, high energy phosphates and myocardial metabolism via lactate flux and  $MVO_2$ . These measurements are done both before and after a period of cross clamp induces ischemia with correction of the shunt. Studies are incomplete in the above measurements both before and after operation. In this way we hope to gain some insight into the mechanisms responsible for the reported higher morbidity and mortality often reported in children with cyanotic congenital heart disease.

f55



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02791-01 SU

## PERIOD COVERED

October 1, 1987 thru September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Hemodynamics and acid-base status during surface-induced hypothermia in the dog.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Julie A. Swain, M.D., Senior Investigator, Surgery Branch, NHLBI

Thomas J. McDonald Jr., B.S., Research Assistant, Surgery Branch, NHLBI

Robert C. Robbins, M.D., Medical Staff Fellow, Surgery Branch, NHLBI

Victoria Hampshire, V.M.D., Veterinarian, Private Practice

## COOPERATING UNITS (if any)

NONE

## LAB/BRANCH

Surgery Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

0.5

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Hypothermia is routinely used during cardiac surgical procedures. In addition, accidental hypothermia is encountered in trauma, exposure, and during operations with open body cavities. Hypothermia alters every hemodynamic, metabolic, and gas transfer parameter. Our laboratory and clinical research is aimed at lessening the deleterious effects of hypothermia and of enhancing the beneficial changes produced by hypothermia. The present study was designed to evaluate the effects of two different pH management schemes (alpha-stat and pH-stat) on systemic hemodynamics, myocardial function, and systemic metabolism in the dog during hypothermia. Previous investigators have suggested that the alpha-stat scheme might lead to increased metabolism during hypothermia, thus negating the beneficial decrease in oxygen consumption produced by hypothermia.

Dogs were surface-cooled to 26°C and hemodynamic and metabolic parameters measured. The results were that the only difference between alpha-stat and pH-stat animals was that the mean arterial pressure during hypothermia was lower in the alpha-stat animals. Systemic oxygen consumption was no different between the groups and the  $Q_{10}$  was not different. The base excess was increased in the alpha-stat group. There was no lactate production in either group.

These results show that there are few hemodynamic or metabolic differences between moderate hypothermia in surface-cooled dogs during alpha-stat or pH-stat blood pH management. In particular, the pH-stat strategy does not produce a greater decrease in metabolism than the alpha-stat pH strategy during hypothermia. Oxygen delivery to the tissues is adequate with either pH strategy. Experimental and clinical evidence from this laboratory supports the use of alpha-stat pH management during hypothermia.

fsh





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02792-01-SU

## PERIOD COVERED

October 1, 1987 thru September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders)

The effect of pH on whole body metabolism during hypothermic cardiopulmonary bypass.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Julie A. Swain, M.D., Senior Investigator, Surgery Branch, NHLBI

Benjamin Schneider, Cardiopulmonary Perfusionist, Surgery Branch, NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Surgery Branch

## SECTION

3

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

0.25

## PROFESSIONAL:

0.25

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In cardiac surgery, systemic hypothermia is used with cardiopulmonary bypass for myocardial protection and preservation of function. Marked changes occur in organ perfusion and metabolism with hypothermia. The hypothesis of this experimental protocol is that arterial pH is a major determinant of systemic oxygen consumption and anaerobic metabolism during hypothermia cardiopulmonary bypass and of the total body carbon dioxide content in the post-bypass period.

The present experimental protocol was designed to measure systemic oxygen consumption, and the metabolic acid-base status of patients undergoing hypothermic cardiopulmonary bypass for cardiac surgical procedures. Systemic and venous blood samples for blood gases and lactate concentrations and cardiac output or cardiopulmonary bypass flow were measured prior to, during, and after cardiopulmonary bypass in forty patients. The patients were randomly assigned to one of two groups. The blood pH during hypothermia was kept constant at 7.4 in one group (pH-stat). In the other, the pH was increased 0.015 pH units for each one degree decrease in temperature (alpha-stat).

Results show that a uniformly low operative mortality was obtained with either pH scheme. The data on oxygen consumption and lactate production is in the process of analysis.

fb1



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02793-01 SU

## PERIOD COVERED

October 1, 1987 thru September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Effect of Hypothermia on Sheep Brain Energy State and Intracellular pH.

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Julie A. Swain, M.D., Senior Investigator, Surgery Branch, NHLBI

Robert S. Balaban, Ph.D., Chief, Laboratory of Cellular Energetics, NHLBI

Robert C. Robbins, M.D., Medical Staff Fellow, Surgery Branch, NHLBI

Thomas J. McDonald Jr., B.S., Research Assistant, Surgery Branch, NHLBI

## COOPERATING UNITS (if any)

Laboratory of Cellular Energetics

## LAB/BRANCH

Surgery Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

.75

## OTHER:

.25

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Hypothermic cardiopulmonary bypass is commonly used during cardiac surgery. The metabolic and biochemical effects of hypothermia remain poorly understood. Cerebral function during and after cardiopulmonary bypass is an important area of interest in cardiac surgical research. Cerebral dysfunction is a major cause of morbidity and mortality after cardiac operations. The present study was designed to determine the effect of hypothermia on the energy state and the intracellular pH of the brain.

Adolescent sheep were studied with <sup>31</sup>P NMR spectroscopy during both surface-induced hypothermia and during profound hypothermia produced by cardiopulmonary bypass. Preliminary results show that there is no difference in the brain energy state during hypothermia. The intracellular pH closely paralleled the blood pH. No evidence of cellular acidosis was seen, thus implying that cerebral oxygen delivery was adequate.

This study has important clinical implications for hypothermic patients. Cerebral oxygen delivery (as evidenced by preservation of cellular energy charge and lack of acidosis) is adequate both during moderate surface-induced hypothermia and during profound hypothermia produced by cardiopulmonary bypass.

f63



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02794-01 SU

## PERIOD COVERED

October 1, 1987 thru September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Role of Chordae Tendinae in Palliation of Mitral Regurgitation

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Hani A. Hennein, M.D., Principal Investigator, Surgery Branch, NHLBI

Christopher D. Stone, M.D., Medical Staff Fellow, Surgery Branch, NHLBI

Julie A. Swain, M.D., Senior Investigator and Surgeon, Surgery Branch, NHLBI

Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

## COOPERATING UNITS (if any)

Pathology Branch, NHLBI

Nuclear Cardiology Branch, Nuclear Medicine

## LAB/BRANCH

Surgery Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

To assess the relative contribution of the chordal structures and mitral valve apparatus to left ventricular function (LVF), in the presence of chronic mitral regurgitation (CMR), 18/26 animal models of CMR were developed by creating a 4mm perforation of the anterior mitral valve leaflet during cardiopulmonary bypass. Complete hemodynamic, ultrasonographic, and radionuclide functional analysis will be performed on a control group of animals and in all survivors of the perforation operation to evaluate the effects of CMR on LVF.

Data acquired to date show that a 70-80% survivorship was attained in 20-25kg animals, and over 90% in 25-30kg animals. Acute changes in LVF included a drop in  $dp/dt$ , while cardiac output remain unchanged. Right heart, left atrial and wedge pressure increased.

The second phase of the study consists of performing mitral valve replacement (MVR) in 18 control animals, half of which will be randomized to chordal preservation and half to standard native-valve-removal. The study arm consists of randomization of the CMR animals to these same two groups. Studies of LV function and geometry along with nuclear angiographic data will be obtained and analyzed, to assess whether chordal preservation during MVR has any effect on subsequent LVF in normal volume overloaded ventricles.

f65



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02795-01 SU

## PERIOD COVERED

October 1, 1987 thru September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders)

Thyroid Hormone Concentrations and Cardiac Surgery

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Richard E. Clark, M.D., Principal Investigator, Surgery Branch, NHLBI

Robert Groom, B.S., CCP, Surgery Branch, NHLBI

Ronald Edin, M.D., Clinical Pathology, CC

Bruce Weintraub, M.D., NIDDK, MCNE

## COOPERATING UNITS (if any)

Clinical Pathology

LAB/BRANCH

Surgery Branch

SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

0.2

## PROFESSIONAL:

0.1

## OTHER:

0.1

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The hypothesis tested in this study was that thyroid hormone levels during and after cardiopulmonary bypass (CPBP) would be a function of the mode of flow: steady state versus pulsatile. Three blood samples prior to, during, and after CPBP were used. 25 patients 12 in the pulsatile group were used. The data show that TSH increases during hypothermic hemodilutional CPBP free  $t_4$  remained constant, TBG decreased and recovered to 75% of the pre CPBP by 24 hours as did total  $T_4$ . Total  $T_8$  decreased 20% of pre CPBP and remained low for the first 24 hours after operation. Albumin used as a marker of the degree of hemodilution decreased to 55% of pre CPBP levels during CPBP. Pulsatile flow had two effects only a) a transient rise in TSH levels at the mid CPBP interval and b) a lack of increase in albumin concentration after surgery. The latter effect was caused by an increased use of crystalloid solution in the initial post operative in this group. The conclusions were that total  $T_3$  remains at low levels and may contribute to low cardiac output and pulsativity per se has little effect on the concentrations of thyroid hormones.

567





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02796-01-SU

## PERIOD COVERED

October 1, 1987 thru September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Myocardial Preservation with Prostaglandin Bx

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Marc E. Mitchell, M.D., Principle Investigator, Surgery Branch, NHLBI

Robert C. Robbins, M.D., Medical Staff Fellow, Surgery Branch, NHLBI

Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

## COOPERATING UNITS (if any)

NONE

LAB/BRANCH

Surgery Branch, NHLBI

SECTION

## INSTITUTE AND LOCATION

National Heart, Lung and Blood Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

3.5

PROFESSIONAL:

2

OTHER:

1.5

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Prostaglandin Bx (PGBx) is an oligomer of Prostaglandin B1. The dimer and trimer have been shown to protect various tissues, including hepatic, renal, cerebral, and cardiac, from loss of function after ischemia. It is thought that the drug stabilizes mitochondrial membranes, and protects the mitochondria from loss of oxidative phosphorylation.

A total of 85 rat hearts have been given Prostaglandin Bx (PGBx) during ischemia while on the isolated heart apparatus. Each heart received between 40-200 micrograms of PGBx, either at the beginning of the ischemic interval or at the time of reperfusion. The ischemic time was 27 minutes at 37.5°C. The control group received no PGBx. Forty-six percent of the control hearts survived the ischemic period. The treated hearts had survival rates ranging from 38% to 100%. The difference was significant in only one of the treated groups. There were no differences in the percent recovery of systolic pressure, aortic flow, cardiac output or stroke volume between the control and treated groups. The treated animals had a significantly lower recovery of heart rate compared to the treated animals. The conclusion was that PGBx had little if any myocardial protective effect.

J69



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02797-01 SU

## PERIOD COVERED

October 1, 1987 thru September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders)

Energy Spectra of Cardiac Sounds

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Richard E. Clark, M.D., Principle Investigator, Surgery Branch, NHLBI

Gail E. Greenberg, Statistical Assistant, Surgery Branch, NHLBI

David Caden, Medical Instrumentation Technologist, Surgery Branch, NHLBI

Moshe E. Mehlman, Clinical Engineer, Surgery Branch, NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Surgery Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

0.4

## PROFESSIONAL:

0.1

## OTHER:

0.3

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

More than 400 patients have had phonocardiography using a high frequency microphone and accelerometer system. The input is digitized and fast transform fourier analysis applied to the wave form. Energy in dB versus frequency plots are obtained as are impact or closure sounds to determine synchrony of occluder or leaflet closure. Signatures of normal opening and closing sounds have been obtained. The data were obtained from porcine valves (71 aortic, 124 mitral) and mechanical valves (138 aortic, 58 mitral). More than 100 patients have had the sounds of their prosthetic device measure more than once. Analysis have shown for the aortic porcine that the peak frequency of the systolic ejection murmur is tightly correlated to the frequency of maximal closure energy more than 20 valves have been shown to be dysfunctional both by energy spectra analysis and cardiac catheterization studies. Replacement of the fatigued prosthetic valve has been performed in most cases. Pathologic studies confirm marked leaflet calcification and fatigue. Several more years of tracking the same patient will be required before the predictive value of acoustic energy spectra recording can be established as a screening method for patients with bioprosthetic valves.

871



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02798-01 SU

## PERIOD COVERED

October 1, 1987 thru September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Effects of Digoxin &amp; Ischemia on the Immature Myocardium

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Michael D. Crittenden, M.D., Senior Staff Fellow, Surgery Branch, NHLBI

Marc E. Mitchell, M.D., Medical Staff Fellow, Surgery Branch, NHLBI

Gerald Kelly, Research Assistant, Surgery Branch, NHLBI

Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

## COOPERATING UNITS (if any)

Chemistry Department, National Bureau of Standards, Gaithersburg, MD

## LAB/BRANCH

Surgery Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung and Blood Institute, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

0.4

## PROFESSIONAL:

0.1

## OTHER:

0.3

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Newborns with congestive heart failure are universally treated with digitalis compounds. The hypothesis tested in these studies is that this drug is deleterious in the setting of ischemia and reperfusion because of Na/k. ATPase inhibition of abnormally low concentrations of this enzyme. Newborn pigs will be treated with digoxin for five days in clinically relevant doses. Treated and non-treated hearts will have intracellular myocardial sodium and calcium concentrations determine. Three groups of 20 hearts each will be placed on an isolated working heart apparatus. Ischemia and reperfusion will be used and contractility functions assessed together with biochemical analyses. Age matched non-treated controls will be used.



ANNUAL REPORT OF THE  
LABORATORY OF TECHNICAL DEVELOPMENT

OCTOBER 1, 1987 to SEPTEMBER 30, 1988

Fluorescence Spectroscopy

The kinetics of release of fluorescent dye from liposomes due to the lytic action of peptides such as melittin and mastoparan was studied, and a method for assaying lytic compounds was developed. The method is more convenient and sensitive than hemolysis for detecting and quantitating lysis. A survey of peptides found that liposome lysis was not necessarily correlated with mast cell degranulating activity, because peptides could contain either, both, or neither of these activities.

Further evaluation of National Bureau of Standards solid phase fluorescence standards was carried out, using a second generation set of standards. Current work involves measuring the corrected emission spectra of different phosphors imbedded in sintered polytetrafluoroethylene strips.

Luminescence Spectroscopy

The fluorescence properties of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have been studied in order to probe the conformational changes which occur in different regions of the protein with denaturation. Using time-resolved spectroscopy with a picosecond, tunable dye-laser excitation system, we could resolve the tryptophan emission into 3 components associated with 3 different lifetimes. A fluorescent photoproduct attached to the active site served as a probe for this region. The data were consistent with sequential changes in different regions of the protein as a function of time of denaturation in guanidine hydrochloride. Time-resolved anisotropy studies showed changes in the rotational mobility of the photoproduct during denaturation.

The mechanism of concentration quenching of 6CF in liposomes was confirmed to be due to energy transfer to nonfluorescent dimers. Energy transfer calculations together with concentration depolarizing measurements showed that this mechanism could account for all the quenching, and that any component of collisional deactivation was improbable.

A study of the site of excited state protonation of serotonin is under way using ultraviolet irradiation and NMR.

Time Resolved Fluorescence Spectroscopy

A new time-resolved fluorescence facility was developed to provide rapid collection and analysis of fluorescence data related to macromolecular size,





flexibility, folding and fluctuation. The ability to collect multifrequency phase/modulation data (contemporaneously with our state of the art pulse decay system) was added to our facility, as was the capability to observe fluorescence under 3K bar pressures (to examine volume dependences of folding and subunit-subunit affinities of proteins, along with free volume dependence of lipid fluctuations.

The main time-resolved spectrofluorometer was utilized to study the structure and dynamics of many different proteins, including: gramicidin, a "pore-forming" peptide; tubulin, a cytoskeletal component whose pre-filamentous state can be discerned with bound Nile red; arginase and OTCase, enzymes whose linked metabolic feedback is mediated by clear conformational changes identified on our equipment; enzyme I of the phosphotransferase system (work in collaboration with M. Han that led to his being awarded the Lampport Award from the Biophysical Society), a protein whose ligand-dependent subunit association and sulfhydryl reactivity are revealed by nanosecond fluorescence spectra.

Protein folding continued to grow as a priority topic in our lab. The metal-stabilized structures of arginase, alcohol dehydrogenase, glyceraldehyde phosphate dehydrogenase, and other proteins were perturbed so our rapid-collection instrument could chronicle structural change vs. time.

We also continued our inquiry into lipid packing fluctuations, using a unique probe (coronene) that is sensitive to submicrosecond gel fluid equilibration in membranes.

### Biophysical Instrumentation

An all tantulum stopped-flow microcalorimeter, previously reported [Biophysical Journal 49(1986) p.87a; 51(1987) p.443a] has now been used extensively to study the binding enthalpies of DNA-drug interactions. Decomposition of the measured thermogram yields reconstructed thermograms with a time constant of approximately three seconds. Two schemes have been used to reconstruct the measured thermograms. The finite element method of Davids and Berger [J. Biochem & Biophys Methods. 6(1982) p.205-217] requires a knowledge of the physical construction of the calorimeter. The iterative method adapted by Schuette and Mudd [J. Biochem & Biophys Methods, 14 (1987) p. 167-175] requires only that the impulse response be known and digitized at the same rate as the measured thermogram. Results employing both deconvolution methods will be presented. The microcalorimeter is capable of measuring binding enthalpies of 30 microjoules with a standard deviation of 3 microjoules. A reaction requires 80 microliters of each reagent and is completed within 200 seconds thus allowing a typical throughput of 120-150 runs per day. The high resolution of this instrument has permitted accurate measurement of reaction heats at extremely dilute reagent concentrations thereby precluding the need to correct the binding enthalpies for drug and/or DNA aggregation.

The development of a blood substitute, while of primary concern to the Department of Defense for Combat Victims, has important implications for the civilian population since it can be sterilized and rendered virus free. Scale up to 50 liters per run is being started at the Division of Blood Resources, Letterman Army Institute of Research under Col. Robert Winslow. The



development of various instruments, for the biophysical study of hemoglobin began in this section a number of years ago, are now in demand at NIH, LAIR, and various university laboratories. The oxygen equilibrium curve analyses we have been developing with the considerable assistance of Horace Cascio, BEIB Electrical Engineering, and the Fabrication Section of BEIB, has been finished and is presently being debugged. Utilizing summer personnel, we plan to thoroughly test it on  $\text{HbA}_0$  furnished by Letterman. Effects of temperature and chloride binding to hemoglobin on the equilibrium curve will be studied both manometrically and spectrometrically to establish a standard curve. A calorimetric determination of the heats of binding will be done with C. Mudd, BEIB Clinical Engineering.

New advances in Near InfraRed Spectroscopy have led to the development of a system for identifying ATP, ADP, and  $\text{PO}_4^-$  in a mixture with a buffer, such as Bis-Tris, and salts such as KCl. Preliminary work done this past year has permitted us to identify two lines for each compound when done singly, with a 99 % correlation. The next step is to attempt to have as high a correlation in mixtures. The present spectrometer permits scanning any octave, i.e. 600 to 1200, 900 to 1800, 1200 to 2400 nm in 200 milliseconds. For the above work 50 scans were used. Thus we would be limited to a time resolution of 10 seconds. For kinetics studies 10 milliseconds or better is required. We are presently exploring the possibility of utilizing lasing diodes for such work. These have  $10^5$  greater light intensity and a time resolution of nanoseconds.

#### Clinical Devices

A new method for producing a rapid pressure pulse to the thighs has been shown to produce an increase in the diastolic run off pressure to effect circulatory support similar to that produced by cuff inflation with fluids that require bulky machines and a heavy pressure box.

The novelty is the use of elastic recoil of rubber to obtain a rapid compression and a reexpansion of the rubber and a rigid half tube. The thighs are each enclosed by two tapered half tubes that hold the rubber against the walls by atmospheric pressure. To compress the thighs air is admitted to the evacuated space and the energy stored in the stretched rubber produces rapid compression. The R wave keyed with delay produces ear sphygmographic pulses indicating diastolic augmentation of flow with compensatory changes in the pulse wave indicative of effective cardiac support function.

Application to volunteers instrumented with ear plethysmographic sensors has demonstrated that pressure waves equivalent to those produced by pressure activated counterpulsation devices are conveniently and comfortably applied by our elastic recoil system. A protocol for application to patients with indwelling catheters is for confirmation of efficacy is in progress.

Thermal angioplasty is a newly developed technique that employs a heated metallic tip to thermally ablate atherosclerotic plaques for recanalization of obstructed peripheral arterial blood vessels. However, the current Laser Thermal probe employs a high power continuous argon laser which is large in size and high in cost. Last year, we developed an electrically heated thermal tip catheter as a possible alternative to the laser probe, and preliminary testing of this device in an in vivo animal model appears promising.



Another economical method of heating a metallic tip is to harvest the chemical energy from the combustion of hydrogen gas. This energy can be released and harvested inside a catheter tip in a safe manner by utilizing a palladium sponge catalyst which initiates and maintains the chemical combustion in a controlled fashion. A prototype catalytic thermal tip catheter has been designed and fabricated for in vitro testing. A temperature feed back control device has been added to avoid excessive tissue heating with the aim of minimizing the incidence of vessel wall perforation. A commercialized model of the catalytically heated tip has been supplied and evaluated in several in vivo animal models. A human test application is anticipated.

### Microtools

A method for the direct injection of DNA into a relatively large number of cells was conceived and the apparatus for construction of the microglassware was constructed. The idea that cells could be conducted through microchannels, to a trap position where they would be held, automatically injected, then released for culture individually requires a method of constructing the channels, microinjectors and fluid moving devices. A glass lathe with micromanipulation capabilities was constructed and the idea of working quartz with the spread flame confirmed the feasibility of making the micro "glassware" required.

### Separation Science Instrumentation

#### Cross-Axis Synchronous Flow-Through Coil Planet Centrifuge

The second prototype of the cross axis synchronous flow through coil planet centrifuge with a 20 cm revolutionary radius was constructed to study its capability of performing preparative countercurrent chromatography.

A series of preliminary studies on retention of stationary phase and partition efficiency was conducted with a short coil prepared from 0.26 cm I.D. PTFE (polytetrafluoroethylene) tubing. The results indicated what 1) the present system provides satisfactory retention of the stationary phase for various two phase solvent systems with a broad spectrum in hydrophobicity, 2) the lateral coil position on the column holder produces substantially higher retention of the stationary phase than the central coil position, if the proper mode of planetary motion is chosen, and 3) partition efficiencies of test samples expressed in terms of peak resolution were closely correlated with the retention of the stationary phase (correlation coefficient = + 0.8).

Using a pair of large multilayer coil columns connected in series with a total capacity of 1600 ml, preparative capability of the present countercurrent chromatographic method was successfully demonstrated on separation of various samples including dinitrophenyl amino acids, dipeptides, indole auxins, flavonoids (from a crude extract of sea buckthorn), and steroids (from a crude reaction mixture).



### Analytic High-Speed Countercurrent Chromatography with Coil Planet Centrifuge

Analytic high-speed countercurrent chromatography (HSCCC) was successfully applied to separations of various natural products which include flavonoids from sea buckthorn, alkaloids from *Stephania tetrandra* S. Moore, hydroxyanthraquinone derivatives from rheum, etc. The results indicated that 1.) efficient separations of multiple components present in a crude extract can be effected in a short period of time by using the normal and reversed elution modes in succession without interrupting the centrifuge run. 2.) With chloroform solvent systems, which provide a large difference in density between the two phases, the flow rate can be increased up to 5 ml/min without affecting peak resolution, thus achieving efficient separations in less than 15 minutes as in analytical HPLC. 3.) In analogy to the LC/MS system, HSCCC can be effectively interfaced with a mass spectrometer using a thermospray capillary tube device.

### Multistage Mixer Settler Planetary Centrifuge for Countercurrent Chromatography

We have developed a novel method for separating macromolecules on the basis of two-phase liquid partitioning. A rotary-seal-free planetary centrifuge holds a separation column which consists of multiple partition units connected in series with transfer tubes. In the cavity of each partition unit the transfer tube extends to form a mixer which vibrates to stir the contents under an oscillating force field generated by the planetary motion of the centrifuge. Consequently, solutes locally introduced at the inlet of the column are subjected to an efficient partition process in each partition unit and separated according to the partition coefficients. The mixer tube equipped with a flexible silicone rubber joint was found to produce excellent results for partition with viscous polymer phase systems. The capability of the method was demonstrated on the separation of cytochrome C and lysozyme using a PEG/K<sub>2</sub>HPO<sub>4</sub> aqueous/aqueous solvent system.

### Pulmonary and Cardiac Assist Devices

Using animal models, we have shown that mechanical pulmonary ventilation as now practiced can under certain conditions become highly injurious, and lethal (adult respiratory distress syndrome = ARDS). This injury process is related to peak inspiratory pressures, and duration of mechanical pulmonary ventilation.

Using such an animal model of ARDS we have managed sheep on extracorporeal veno-venous blood exchange, while discontinuing all mechanical pulmonary ventilation. Within 24 hours of change in treatment to extracorporeal blood gas exchange, there was rapid improvement in all animals, and 9/11 sheep were weaned off bypass and to room air. In a control group of animals treated with conventional continuous positive pressure ventilation (CPP) only 3/11 sheep could be weaned off bypass.

This animal model of ARDS can become sufficiently severe so that no form of presently available therapy will lead to survivors. Such understanding only emphasizes the as yet unrecognized dangers implied in the use of CPPV.





## Cell Culture and Calcium Measurement Medium

Use of the porous bottom culture dishes (PBCDs) developed in this laboratory for the study of epithelial cells grown in confluent layers continues to grow. This growth represents increased use in more laboratories world-wide and also their application to ever increasing numbers of cell types. This large utilization has resulted in part from the fact that PBCDs are now commercially available from 5 sources (Costar Corp., Funakoski Pharmaceutical Co., ICN Biomedicals, Inc., Millipore Corp., Nuclepore Corp.) with several types of porous membranes, some with cell culture treatments and coatings. The objectives of the research projects being undertaken with these PBCDs go far beyond anything we had in mind at the outset of our development of them.

The PBCDs made with collagen membranes also made in this laboratory still provide better optical properties (for phase microscopy) than most of the "homemade" and commercial PBCDs. Many cell types grow well on the collagen also. In fact, we have grown endothelial cells on one side of the collagen membrane and smooth muscle cells on the other side. This makes a good model of a blood vessel. A type of membrane we are developing to have controlled permeability has even better optical properties than our collagen membranes. This membrane material is reconstituted cellulose to which we give a surface treatment to facilitate cell attachment.

The major role play by Ca in the regulation of many cellular processes has stimulated us to try to simplify methods for measuring free Ca activity, so that they will be no more difficult than pH measurements. The use of hydrophobic porous membrans and neutral carrier Ca sensors has resulted in a 2 mm diameter electrode which has rapid response and low resistance. These characteristics allow such electrodes to be used with many pH meters.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01404-20 LTD

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Membrane Lungs for Long Term Respiratory, Cardiac and Cardiorespiratory Assist

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	T. Kolobow	Medical Officer	LTD:NHLBI
	G. Foti	Visiting Fellow	LTD:NHLBI
	F. Rossi	Visiting Fellow	LTD:NHLBI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Technical Development

## SECTION

Section on Pulmonary and Cardiac Assist Devices

## INSTITUTE AND LOCATION

NHLBI NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

3

## PROFESSIONAL:

3

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Using animal models, we have shown that mechanical pulmonary ventilation as now practiced can under certain conditions become highly injurious, and lethal (adult respiratory distress syndrome = ARDS). This injury process is related to peak inspiratory pressures, and duration of mechanical pulmonary ventilation.

Using such an animal model of ARDS we have managed sheep on extracorporeal veno-venous blood gas exchange, while discontinuing all mechanical pulmonary ventilation. Within 24 hours of change in treatment to extracorporeal blood gas exchange, there was rapid improvement in all animals, and 9/11 sheep were weaned off bypass and to room air. In a control group of animals treated with conventional continuous positive pressure ventilation (CPP) only 3/11 sheep could be weaned off bypass.

This animal model of ARDS can become sufficiently severe so that no form of presently available therapy will lead to survivors. Such understanding only emphasizes the as yet unrecognized dangers implied in the use of CPPV.

882



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01407-25 LTD

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Luminescence Spectroscopy in Biomedical Research

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	Raymond F. Chen	Sr. Investigator	LTD:NHLBI
	Jay R. Knutson	Sr. Staff Fellow	LTD:NHLBI
	Chen-Lu Tsou	Fogarty Scholar	NIH

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Technical Development

## SECTION

## INSTITUTE AND LOCATION

NHLBI NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

0.7

## PROFESSIONAL:

0.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (h) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The fluorescence properties of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have been studied in order to probe the conformational changes which occur in different regions of the protein with denaturation. Using time-resolved spectroscopy with a picosecond, tunable dye-laser excitation system, we could resolve the tryptophan emission into 3 components associated with 3 different lifetimes. A fluorescent photoproduct attached to the active site served as a probe for this region. The data were consistent with sequential changes in different regions of the protein as a function of time of denaturation in guanidine hydrochloride. Time-resolved anisotropy studies showed changes in the rotational mobility of the photoproduct during denaturation.

The mechanism of concentration quenching of 6CF in liposomes was confirmed to be due to energy transfer to nonfluorescent dimers. Energy transfer calculations together with concentration depolarization measurements showed that this mechanism could account for all the quenching, and that any component of collisional deactivation was improbable.

A study of the site of excited state protonation of serotonin is under way using ultraviolet irradiation and NMR.

887



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 HL 01408-23 LTD
PERIOD COVERED October 1, 1987 to September 30, 1988		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Methods in Fluorescence Spectroscopy		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)  <div style="display: flex; justify-content: space-between;"> <span>P.I. Raymond F. Chen</span> <span>Sr. Investigator</span> <span>LTD:NHLBI</span> </div>		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Technical Development		
SECTION		
INSTITUTE AND LOCATION NHLBI NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 0.5	PROFESSIONAL: 0.5	OTHER:
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <span><input type="checkbox"/> (a) Human subjects</span> <span><input type="checkbox"/> (b) Human tissues</span> <span><input checked="" type="checkbox"/> (c) Neither</span> </div> <div style="display: flex; justify-content: space-between;"> <span><input type="checkbox"/> (a1) Minors</span> <span><input type="checkbox"/> (a2) Interviews</span> </div>		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>The kinetics of release of fluorescent dye from liposomes due to the lytic action of peptides such as melittin and mastoparan was studied, and a method for assaying lytic compounds was developed. The method is more convenient and sensitive than hemolysis for detecting and quantitating lysis. A survey of peptides found that liposome lysis was not necessarily correlated with mast cell degranulating activity, because peptides could contain either, both, or neither of these activities.</p> <p>Further evaluation of National Bureau of Standards solid phase fluorescence standards was carried out, using a second generation set of standards. Current work involves measuring the corrected emission spectra of different phosphors imbedded in sintered polytetrafluoroethylene strips.</p>		

892





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01413-26 LTD

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Development of Biophysical Methods for Studying Bio-Macromolecular Reactions

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P. I. R. L. Berger Chief, Biophysical Instrumentation Section LTD:NHLBI

## COOPERATING UNITS (if any)

NIA, Lab. Molecular Biology (J. Froehlich); U. of Penn. (L. Thiebault); DRS, Biomedical Engineering &amp; Instrumentation Branch (H. Cascio); Commonwealth Technology, Alexandria, VA; Div. Blood Resources, LAIR (R. Winslow).

## LAB/BRANCH

Laboratory of Technical Development

## SECTION

Biophysical Instrumentation Section

## INSTITUTE AND LOCATION

NHLBI NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

3

## PROFESSIONAL:

2

## OTHER:

1.0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The development of a blood substitute, while of primary concern to the Dept. of Defense for Combat Victims, has important implications for the civilian population since it can be sterilized and rendered virus free. Scale up to 50 l/run is being started at the Div. of Blood Resources, Letterman Army Inst. of Research under Col. Robert Winslow. The development of various instruments, for the biophysical study of hemoglobin begun in this section many years ago, are now in demand at NIH, LAIR, and various univ. labs. The oxygen equilibrium curve analyses we have been developing with the considerable assistance of Horace Cascio, BEIB Elec. Eng., and the Fabrication Section of BEIB, has been finished and is presently being debugged. Utilizing summer personnel, we plan to thoroughly test it on HbAo furnished by LAIR. Effects of temperature and chloride binding to hemoglobin on the equilibrium curve will be studied both manometrically and spectrometrically to establish a standard curve. A calorimetric determination of the heats of binding will be done with C. Mudd, BEIB Applied Clinical Engineering.

New advances in Near InfraRed Spectroscopy have led to the development of a system for identifying ATP, ADP, and PO<sub>4</sub> in a mixture with a buffer, such as Bis-Tris, and salts such as KCl. Preliminary work has permitted us to identify two lines for each compound when done singly, with 99% correlation. The next step is to attempt to have as high a correlation in mixtures. The present spectrometer permits scanning any octave, i.e. 600 to 1200, 900 to 1800, 1200 to 2400 nm in 200 msec. For the above work, 50 scans were used. We would be limited to a time resolution of 10 sec. For kinetics studies 10 msec. or better is required. We are presently exploring utilizing lasing diodes for such work. These have 10(5th) greater light intensity and a time resolution of nanoseconds.

f95



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01414-16 LTD

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Development of Biocalorimeters for Solution and Cell Biochemical Studies

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P. I. R. L. Berger Chief, Biophysical Instrumentation Section LTD:NHLBI  
Other: C. P. Mudd Biomedical Engineer BEIB:DRS

Cooperating Units: Richard Shrager, DCRT, NIH; M. Marini, Div. of Blood

## COOPERATING UNITS (if any)

Resources, LAIR; N. Davids, Penn State U.; Commonwealth  
Scientific, Alexandria, VA; Commonwealth Technology, Inc. Alexandria, VA.; K.  
Breslauer, Dept. of Chem., Rutgers U.; David Remeta, Dept. of Chem., Rutgers U.

## LAB/BRANCH

Laboratory of Technical Development

## SECTION

Biophysical Instrumentation Section

## INSTITUTE AND LOCATION

NHLBI NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

3.0

## PROFESSIONAL:

3.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

An all tantulum stopped-flow microcalorimeter, previously reported [Biophysical Journal 49(1986) p.87a; 51(1987) p.443a], has now been used extensively to study the binding enthalpies of DNA-drug interactions. Decomposition of the measured thermograms yields reconstructed thermograms with a time constant of approximately three seconds. Two schemes have been used to reconstruct the measured thermograms. The finite element method of Davids and Berger [J. Biochem & Biophys Methods. 6(1982) p.205-217] requires a knowledge of the physical construction of the calorimeter. The iterative method adapted by Schuette and Mudd [J. Biochem & Biophys Methods, 14 (1987) p. 167-175] requires only that the impulse response be known and digitized at the same rate as the measured thermogram. Results employing both deconvolution methods will be presented. The microcalorimeter is capable of measuring binding enthalpies of 30 microjoules with a standard deviation of 3 microjoules. A reaction requires 80 microliters of each reagent and is completed within 200 seconds thus allowing a typical throughput of 120-150 runs per day. The high resolution of this instrument has permitted accurate measurement of reaction heats at extremely dilute reagent concentrations thereby precluding the need to correct the binding enthalpies for drug and/or DNA aggregation.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 HL 01421-13 LTD
PERIOD COVERED October 1, 1987 to September 30, 1988		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Development of Electrochemical and Physiological Methods for Cell Research		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
P.I.	R. E. Steele	Physical Science Investigator
Others:	R. L. Bowman	Chief, LTD
	H. S. Kruth	Senior Investigator
	J. P. Johnson	Chief, Nephrology
		LTD:NHLBI
		LTD:NHLBI
		EA:NHLBI
		WRAIR
COOPERATING UNITS (if any)		
Laboratory of Experimental Atherosclerosis, NHLBI Department of Nephrology, Walter Reed Army Institute of Research		
LAB/BRANCH Laboratory of Technical Development		
SECTION		
INSTITUTE AND LOCATION NHLBI NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 1.1	PROFESSIONAL: 1.1	OTHER:
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)		
<p>Use of the porous bottom culture dishes (PBCDs) developed in this laboratory for the study of epithelial cells grown in confluent layers continues to grow. This growth represents increased use in more laboratories world-wide and also their application to ever increasing numbers of cell types. This large utilization has resulted in part from the fact that PBCDs are now commercially available from 5 sources (Costar Corp., Funakoski Pharmaceutical Co., ICN Biomedicals, Inc., Millipore Corp., Nuclepore Corp.) with several types of porous membranes, some with cell culture treatments and coatings. The objectives of the research projects being undertaken with these PBCDs go far beyond anything we had in mind at the outset of our development of them.</p> <p>The PBCDs made with collagen membranes also made in this laboratory still provide better optical properties (for phase microscopy) than most of the "homemade" and commercial PBCDs. Many cell types grow well on the collagen also. In fact, we have grown endothelial cells on one side of the collagen membrane and smooth muscle cells on the other side. This makes a good model of a blood vessel. A membrane we are developing to have controlled permeability has even better optical properties than our collagen membranes. This membrane material is reconstituted cellulose to which we give a surface treatment to facilitate cell attachment.</p> <p>The major role played by Ca in the regulation of many cellular processes has stimulated us to try to simplify methods for measuring free Ca activity, so that they will be no more difficult than pH measurements. The use of hydrophobic porous membranes and neutral carrier Ca sensors has resulted in a 2 mm diameter electrode which has rapid response and low resistance. These characteristics allow such electrodes to be used with many pH meters.</p>		

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 HL 01452-05 LTD
PERIOD COVERED October 1, 1987 to September 30, 1988		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Time Resolved Fluorescence Spectroscopy		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)		
P.I.	J. R. Knutson	Sr. Staff Fellow LTD:NHLBI
Other	R. F. Chen	Sr. Investigator LTD:NHLBI
Cooperating units: L. Davenport (CUNY); M. Han (LB:NHLBI, Johns Hopkins Univ.) D. Walbridge, J. Wages, S. Roseman, C. Anfinsen, L. Brand (JHU); D. Sackett,		
COOPERATING UNITS (if any) J. Wolff (NIDDK); S. Green, P. Hensley (Georgetown); Sue Scarlata (Cornell); R. Hirsch (Einstein); C.L. Tsou (China); F. Friedman (C:ETB); D. Manchester, A. Westin (C:LMC); A. Russo (C:ROB); J. Beechem, E. Gratton (UI-U/C); 12 other.		
LAB/BRANCH Laboratory of Technical Development		
SECTION		
INSTITUTE AND LOCATION NHLBI NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 1.25	PROFESSIONAL: 1.25	OTHER:
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)		
<p>A new time-resolved fluorescence facility was developed to provide rapid collection and analysis of fluorescence data related to macromolecular size, flexibility, folding and fluctuations. The ability to collect multifrequency phase/modulation data (contemporaneously with our state of the art pulse decay system) was added to our facility, as was the capability to observe fluorescence under 3K bar pressures (to examine volume dependences of folding and subunit-subunit affinities of proteins, along with free volume dependence of lipid fluctuations.)</p> <p>The main time-resolved spectrofluorometer was utilized to study the structure and dynamics of many different proteins, including: gramicidin, a "pore-forming" peptide; tubulin, a cytoskeletal component whose pre-filamentous state can be discerned with bound Nile red; arginase and OTCase, enzymes whose linked metabolic feedback is mediated by clear conformational changes identified on our equipment; enzyme I of the phosphotransferase system (work in collaboration with M. Han that led to his being awarded the Lamport Award from the Biophysical Society), a protein whose ligand-dependent subunit association and sulfhydryl reactivity are revealed by nanosecond fluorescence spectra.</p> <p>Protein folding continued to grow as a priority topic in our lab. The metal-stabilized structures of arginase, alcohol dehydrogenase, glyceraldehyde phosphate dehydrogenase, and other proteins were perturbed so our rapid-collection instrument could chronicle structural change vs. time.</p> <p>We also continued our inquiry into lipid packing fluctuations, using a unique probe (coronene) that is sensitive to submicrosecond gel fluid equilibration in membranes.</p>		





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01461-03 LTD

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Hot Tip Catheter for Percutaneous Removal of Atherosclerotic Plaque

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P. I.	R. L. Bowman	Chief, LTD	LTD:NHLBI
Others	D. Y. Lu	Clinical Fellow	CB: NHLBI
	M. Leon	Senior Investigator	CB: NHLBI
	L. G. Prevosti	Special Volunteer	CB: NHLBI

## COOPERATING UNITS (if any)

Cardiology Branch

## LAB/BRANCH

Laboratory of Technical Development

## SECTION

## INSTITUTE AND LOCATION

NHLBI NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2

## PROFESSIONAL:

2

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Thermal angioplasty is a newly developed technique that employs a heated metallic tip to thermally ablate atherosclerotic plaques for recanalization of obstructed peripheral arterial blood vessels. However, the current Laser Thermal Probe employs a high power continuous argon laser which is large in size and high in cost. Last year, we developed an electrically heated thermal tip catheter as a possible alternative to the laser probe, and preliminary testing of this device in an in vivo animal model appears promising.

Another economical method of heating a metallic tip is to harvest the chemical energy from the combustion of hydrogen gas. This energy can be released and harvested inside a catheter tip in a safe manner by utilizing a palladium sponge catalyst which initiates and maintains the chemical combustion in a controlled fashion. A prototype catalytic thermal tip catheter has been designed and fabricated for in vitro testing. A temperature feed back control device has been added to avoid excessive tissue heating with the aim of minimizing the incidence of vessel wall perforation. A commercialized model of the catalytically heated tip has been supplied and evaluated in several in vivo animal models. A human test application is anticipated.

909



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01462-02 LTD

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cross-Axis Synchronous Flow-Through Coil Planet Centifuge

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	Yoichiro Ito	Senior Investigator	LTD:NHLBI
Other:	Tian You Zhang	Guest Worker	LTD:NHLBI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Technical Development

## SECTION

## INSTITUTE AND LOCATION

NHLBI NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.7

## PROFESSIONAL:

1.5

## OTHER:

0.2

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The second prototype of the cross axis synchronous flow through coil planet centrifuge with a 20 cm revolutionary radius was constructed to study its capability of performing preparative countercurrent chromatography.

A series of preliminary studies on retention of stationary phase and partition efficiency was conducted with a short coil prepared from 0.26 cm I.D. PTFE (polytetrafluoroethylene) tubing. The results indicated that 1) the present system provides satisfactory retention of the stationary phase for various two phase solvent systems with a broad spectrum in hydrophobicity, 2) the lateral coil position on the column holder produces substantially higher retention of the stationary phase than the central coil position, if the proper mode of planetary motion is chosen, and 3) partition efficiencies of test samples expressed in terms of peak resolution were closely correlated with the retention of the stationary phase (correlation coefficient = + 0.8).

Using a pair of large multilayer coil columns connected in series with a total capacity of 1600 ml, preparative capability of the present countercurrent chromatographic method was successfully demonstrated on separation of various samples including dinitrophenyl amino acids, dipeptides, indole auxins, flavonoids (from a crude extract of sea buckthorn), and steroids (from a crude reaction mixture).



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01463-02 LTD

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Analytic High-Speed Countercurrent Chromatography with Coil Planet Centrifuge

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	Yoichiro Ito	Senior Investigator	LTD:NHLBI
Others:	Tian You Zhang	Guest Worker	LTD:NHLBI
	Lewis K. Pannell	Senior Investigator	LBC:NIDDK

## COOPERATING UNITS (if any)

Pharma-Tech Research Corp., Baltimore, MD. (F.E.Chou)

## LAB/BRANCH

Laboratory of Technical Development

## SECTION

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.2

## PROFESSIONAL:

1.0

## OTHER:

0.2

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Analytic high-speed countercurrent chromatography (HSCCC) was successfully applied to separations of various natural products which include flavonoids from sea buckthorn, alkaloids from *Stephania tetrandra* S. Moore, hydroxyanthraquinone derivatives from rheum, etc. The results indicated:

1. Efficient separations of multiple components present in a crude extract can be effected in a short period of time by using the normal and reversed elution modes in succession without interrupting the centrifuge run.

2. With chloroform solvent systems, which provide a large difference in density between the two phases, the flow rate can be increased up to 5 ml/min without affecting peak resolution, thus achieving efficient separations in less than 15 minutes as in analytical HPLC.

3. In analogy to the LC/MS system, HSCCC can be effectively interfaced with a mass spectrometer using a thermospray capillary tube device.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01464-02 LTD

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

External Counterpulsation with Elastic Recoil

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	R. L. Bowman	Chief, LTD	LTD:NHLBI
Other:	D. Lu	Clinical Fellow	CB:NHLBI
	J. Brush	Special Volunteer	CB:NHLBI

## COOPERATING UNITS (if any)

Cardiology Branch, NHLBI

## LAB/BRANCH

Laboratory of Technical Development

## SECTION

## INSTITUTE AND LOCATION

NHLBI NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.5

## PROFESSIONAL:

1.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A new method for producing a rapid pressure pulse to the thighs has been shown to produce an increase in the diastolic run off pressure to effect circulatory support similar to that produced by cuff inflation with fluids that require bulky machines and a heavy pressure box.

The novelty is the use of elastic recoil of rubber to obtain a rapid compression and a reexpansion of the rubber by evacuating the space between the rubber and a rigid half tube. The thighs are each enclosed by two tapered half tubes that hold the rubber against the walls by atmospheric pressure. To compress the thighs air is admitted to the evacuated space and the energy stored in the stretched rubber produces rapid compression. The R wave keyed with delay produces ear sphygmographic pulses indicating diastolic augmentation of flow with compensatory changes in the pulse wave indicative of effective cardiac support function.

Application to volunteers instrumented with ear plethysmographic sensors has demonstrated that pressure waves equivalent to those produced by pressure activated counterpulsation devices are conveniently and comfortably applied by our elastic recoil system. A protocol for application to patients with indwelling catheters is for confirmation of efficacy is in progress.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01465-01 LTD

## PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Multistage Mixer Settler Planetary Centrifuge for Countercurrent Chromatography

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. Yoichiro Ito

Senior Investigator

LTD:NHLBI

Other: Tian You Zhang

Guest Worker

LTD:NHLBI

## COOPERATING UNITS (if any)

Biomedical Engineering and Instrumentation Branch, Division of Research Services, NIH (W. Groves, J. Slemp)

## LAB/BRANCH

Laboratory of Technical Development

## SECTION

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

0.6

## PROFESSIONAL:

0.5

## OTHER:

0.1

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have developed a novel method for separating macromolecules on the basis of two-phase liquid partitioning.

A rotary-seal-free planetary centrifuge holds a separation column which consists of multiple partition units connected in series with transfer tubes. In the cavity of each partition unit the transfer tube extends to form a mixer which vibrates to stir the contents under an oscillating force field generated by the planetary motion of the centrifuge. Consequently, solutes locally introduced at the inlet of the column are subjected to an efficient partition process in each partition unit and separated according to their partition coefficients. The mixer tube equipped with a flexible silicone rubber joint was found to produce excellent results for partition with viscous polymer phase systems. The capability of the method was demonstrated on separation of cytochrome C and lysozyme using a PEG/K<sub>2</sub>HPO<sub>4</sub> aqueous/aqueous solvent system.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01466-01 LTD

## PERIOD COVERED

October 1, 1987 to September 30, 1988

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cell Injection Using Microglassware

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P. I. R. L. Bowman

Chief, LTD

LTD:NHLBI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Technical Development

## SECTION

## INSTITUTE AND LOCATION

NHLBI NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

0.25

## PROFESSIONAL:

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A method for the direct injection of DNA into a relatively large number of cells was conceived and the apparatus for construction of the micro glassware was constructed.

The idea that cells could be conducted through microchannels, to a trap position where they would be held, automatically injected, then released for culture individually requires a method of constructing the channels, microinjectors and fluid moving devices.

A glass lathe with micromanipulation capabilities was constructed and the idea of working quartz with the spread flame confirmed the feasibility of making the micro "glassware" required.













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